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# CONTENTS OF VOLUME 181

No. 1, NOVEMBER, 1949

	PAGE
HUNDLEY, JAMES M. Influence of fructose and other carbohydrates on the niacin requirement of the rat.....	1
RODERUCK, CHARLOTTE E. Analysis of certain components of skeletal muscle during vitamin E deficiency.....	11
BARBER, MARY ALICE, BASINSKI, DANIEL H., and MATTILL, H. A. Transamination in the muscles of animals deprived of vitamin E.....	17
HESS, W. C. The rates of absorption and the formation of liver glycogen by methionine, cystine, and cysteine.....	23
STETTEN, MARJORIE R. Some aspects of the metabolism of hydroxyproline, studied with the aid of isotopic nitrogen.....	31
MUELLER, J. HOWARD, and MILLER, PAULINE A. Glutamine in the production of tetanus toxin.....	39
LERNER, EDWIN M., and MUELLER, J. HOWARD. The rôle of glutamine in the glucose metabolism of <i>Clostridium tetani</i> .....	43
HERBST, EDWARD J., and SNELL, ESMOND E. Putrescine and related compounds as growth factors for <i>Hemophilus parainfluenzae</i> 7901.....	47
JACOBS, WALTER A., and SATO, YOSHIO. The veratrine alkaloids. XXX. A further study of the structure of veratramine and jervine.....	55
HUNTER, F. EDMUND, JR., and HIXON, WALTER S. Anaerobic phosphorylation due to the dismutation of $\alpha$ -ketoglutaric acid in the presence of ammonia..	67
HUNTER, F. EDMUND, JR., and HIXON, WALTER S. Phosphorylation coupled with the oxidation of $\alpha$ -ketoglutaric acid.....	73
BASS, ALLAN D., and PLACE, ELIZABETH F. Uric acid and allantoin excretion in normal and tumor-bearing mice.....	81
SHAW, ELLIOTT, and WOOLLEY, D. W. A new and convenient synthesis of 4-amino-5-imidazolecarboxamide.....	89
PETERSON, D. H., and REINEKE, L. M. The chemistry of circulin; chromatographic isolation of the amino acid constituents with powdered cellulose.....	95
WINSTEN, WALTER A., and EIGEN, EDWARD. Paper chromatography of vitamin B <sub>12</sub> and related bacterial growth factors.....	109
ASTWOOD, E. B., GREER, MONTE A., and ETTLINGER, MARTIN G. 1-5-Vinyl-2-thiooxazolidone, an antithyroid compound from yellow turnip and from <i>Brassica</i> seeds.....	121
VILLEE, CLAUDE A., and HASTINGS, A. BAIRD. The utilization <i>in vitro</i> of C <sup>14</sup> -labeled acetate and pyruvate by diaphragm muscle of rat.....	131
ZIMMERMAN, WALTER J., and LAYTON, WILLIAM M., JR. A polarographic micro-method for the determination of blood chloride.....	141
PARK, JAMES T., and JOHNSON, MARVIN J. A submicrodetermination of glucose.....	149
SUTHERLAND, EARL W., POSTERNAK, THEODORE, and CORI, CARL F. Mechanism of the phosphoglyceric mutase reaction.....	153
CANN, JOHN R., BROWN, RAYMOND A., and KIRKWOOD, JOHN G. Application of electrophoresis-convection to the fractionation of bovine $\gamma$ -globulin....	161
WOOD, W. A., and GUNSALUS, I. C. Serine and threonine deaminases of <i>Escherichia coli</i> : activators for a cell-free enzyme.....	171

BENEDICT, JEAN D., FORSHAM, PETER H., and STETTEN, DEWITT, JR. The metabolism of uric acid in the normal and gouty human studied with the aid of isotopic uric acid . . . . .	183
TAUSKY, HERTHA H. A microcolorimetric method for the determination of citric acid. II. A note on the substitution of ferrous sulfate for hydrazine sulfate as the reducing agent . . . . .	195
SCHWEIGERT, B. S., and MARQUETTE, MONA M. Further studies on the metabolism of 3-hydroxyanthranilic acid by rat liver slices and homogenates. .	199
KOZLOFF, LLOYD M., and PUTNAM, FRANK W. Biochemical studies of virus reproduction. II. Chemical composition of <i>Escherichia coli</i> bacteriophage T <sub>2</sub> and its host . . . . .	207
STADTMAN, E. R., and BARKER, H. A. Fatty acid synthesis by enzyme preparations of <i>Clostridium kluyveri</i> . V. A consideration of postulated 4-carbon intermediates in butyrate synthesis . . . . .	221
ABRAMS, ADOLPH, COHEN, PHILIP P., and MEYER, OVID O. The physical properties of a cryoglobulin obtained from lymph nodes and serum of a case of lymphosarcoma . . . . .	237
PARK, C. R., and KRAHL, M. E. Effect of pituitary extracts upon glucose uptake by diaphragms from normal, hypophysectomized, and hypophysectomized-adrenalectomized rats . . . . .	247
RICHERT, DAN A., EDWARDS, SALLY, and WESTERFELD, W. W. On the determination of liver xanthine oxidase and the respiration of rat liver homogenates . . . . .	255
PRESCOTT, BLANCHE A., BOREK, ERNEST, BRECHER, ARTHUR, and WAELSCH, HEINRICH. Studies on oligophrenia phenylpyruvica. I. Microbiological determination of L- and D-phenylalanine and of phenyllactic acid . . . .	278
LERNER, AARON BUNSEN. On the metabolism of phenylalanine and tyrosine .	281
HOCH, FREDERIC L., and VALLEE, BERT L. Precipitation by trichloroacetic acid as a simplification in the determination of zinc in blood and its components . . . . .	295
ROSE, WILLIAM C., SMITH, LEONARD C., WOMACK, MADELYN, and SHANE, MORTON. The utilization of the nitrogen of ammonium salts, urea, and certain other compounds in the synthesis of non-essential amino acids <i>in vivo</i> . . . . .	307
BINKLEY, FRANCIS. Enzymatic hydrolysis and oxidation of monothiophosphate . . . . .	317
MACLEOD, LESLIE D. Determination of alcohol by microdiffusion . . . . .	323
KALLO, REINO E., and BERG, CLARENCE P. Tryptophan metabolism. XII. Tryptophan, kynurenine, and related compounds as precursors of nicotinic acid . . . . .	333
NACHLAS, MARVIN M., and SELIGMAN, ARNOLD M. Evidence for the specificity of esterase and lipase by the use of three chromogenic substrates . . . .	343
EVANS, JOHN D., and BIRD, ROBERT M. Metabolism of rabbit bone marrow <i>in vitro</i> in Ringer-bicarbonate medium containing no added glucose . . . .	357
BEINERT, HELMUT, and REISSMANN, KURT R. Studies on the incorporation of injected cytochrome c into tissue cells. I. Injection of non-radioactive cytochrome c into rats previously given radioiron . . . . .	367
DISCHE, ZACHARIAS. Spectrophotometric method for the determination of free pentose and pentose in nucleotides . . . . .	379
SEALOCK, ROBERT RIDGELY, and WHITE, HILDA S. Neopyrithiamine and the thiaminase of fish tissues . . . . .	398

AMBERSON, WILLIAM R., ERDÖS, T., CHINN, BETTY, and LUDS, HANS. Electrophoretic and ultracentrifugal analyses of protein extracted from whole mammalian muscles.....	405
LENNINGER, ALBERT L., and SMITH, SYLVIA WAGNER. Efficiency of phosphorylation coupled to electron transport between dihydrodiphosphopyridine nucleotide and oxygen.....	415
REMMERT, LEMAR F., and COHEN, PHILIP P. Partial purification and properties of a proteolytic enzyme of human serum.....	431

## No. 2, DECEMBER, 1949

EADIE, G. S., BERNHEIM, FREDERICK, and BERNHEIM, MARY L. C. The partial purification and properties of animal and plant hydantoinases.....	449
CLARK, HAROLD W., DOUNCE, ALEXANDER L., and STOTZ, ELMER. An improved method for the extraction and purification of diphosphopyridine nucleotide.....	459
SAUBERLICH, H. E. The effect of folic acid upon the urinary excretion of the growth factor required by <i>Leuconostoc citrovorum</i> .....	467
LORBER, VICTOR, COOK, MARGARET, and MEYER, JOE. Conversion of octanoic acid to rat liver glycogen, studied with C <sup>14</sup> , C <sup>13</sup> -labeled octanoate.....	475
FOOTE, MURRAY W., LITTLE, JOHN E., and SPROSTON, THOMAS J. On naphthoquinones as inhibitors of spore germination of fungi.....	481
WEINHOUSE, SIDNEY, MILLINGTON, RUTH H., and FRIEDMAN, BERNICE. The effect of carbohydrate on the oxidation of fatty acids by liver slices.....	489
FREDERICQ, EUGENE, and DEUTSCH, H. F. Studies on ovomucoid.....	499
STONE, GILBERT C. H., and GOLDZIEHER, JOSEPH W. A rapid colorimetric method for the determination of sodium in biological fluids and particularly in serum.....	511
BARTLETT, PAUL D., and GAEBLER, OLIVER H. Studies on the mechanism of nitrogen storage. II. Effects of anterior pituitary growth hormone preparations on kidney glutaminase.....	523
BARTLETT, PAUL D., and GAEBLER, OLIVER H. Studies on the mechanism of nitrogen storage. III. The effects of anterior pituitary growth hormone preparations on the pyruvate-activated deamidation of glutamine in liver tissue.....	529
PRICE, VINCENT E., MEISTER, ALTON, GILBERT, JAMES B., and GREENSTEIN, JESSE P. The separation of dehydropeptidase and analogous L- and D-peptidases.....	535
FODOR, PAUL J., and GREENSTEIN, JESSE P. Separation of enzymatic activities toward chloroacetylalanine, chloroacetylalanylglycine, and glycylalanine..	549
WILLIAMS, J. N., JR., and ELVEHJEM, C. A. The relation of amino acid availability in dietary protein to liver enzyme activity.....	559
BARKI, V. H., FEIGELSON, P., COLLINS, R. A., HART, E. B., and ELVEHJEM, C. A. Factors influencing galactose utilisation.....	565
BOAS, NORMAN F. Isolation of hyaluronic acid from the cock's comb.....	573
PEARSON, HAROLD E., and WINZLER, RICHARD J. Oxidative and glycolytic metabolism of minced day-old mouse brain in relation to propagation of Theiler's GD VII virus.....	577
RAVELSON, MAX E., JR., WINZLER, RICHARD J., and PEARSON, HAROLD E. The effects of Theiler's GD VII virus on P <sup>32</sup> uptake by minced one day-old mouse brain.....	583

RAFELSON, MAX E., JR., WINZLER, RICHARD J., and PEARSON, HAROLD E. The effects of Theiler's GD VII virus on the incorporation of radioactive carbon from glucose into minced one day-old mouse brain. . . . .	595
FREEDMAN, LEON D., and CORWIN, ALSOPH H. Oxidation-reduction potentials of thiol-disulfide systems . . . . .	601
KAUFMAN, SEYMOUR, and NEURATH, HANS. Structural requirements of specific inhibitors for $\alpha$ -chymotrypsin . . . . .	623
HOFFMANN, C. E., STOKSTAD, E. L. R., HUTCHINGS, B. L., DORNBUSH, A. C., and JUKES, THOMAS H. The microbiological assay of vitamin B <sub>12</sub> with <i>Lactobacillus leichmannii</i> . . . . .	635
WEINHOUSE, SIDNEY, and MILLINGTON, RUTH H. Ketone body formation from tyrosine . . . . .	645
SACKS, JACOB. A fractionation procedure for the acid-soluble phosphorus compounds of liver . . . . .	655
HENDERSON, L. M., and HIRSCH, HERBERT M. Quinolinic acid metabolism. I. Urinary excretion by the rat following tryptophan and 3-hydroxyanthranilic acid administration . . . . .	667
HENDERSON, L. M. Quinolinic acid metabolism. II. Replacement of nicotinic acid for the growth of the rat and <i>Neurospora</i> . . . . .	677
HENDERSON, L. M., and RAMASARMA, G. B. Quinolinic acid metabolism. III. Formation from 3-hydroxyanthranilic acid by rat liver preparations . . . . .	687
LAMPSON, GEORGE P., and LARDY, HENRY A. Phosphoric esters of biological importance. II. The synthesis of glucose-6-phosphate from 1,2-isopropylidene-5,6-anhydro-D-glucofuranose . . . . .	693
LAMPSON, GEORGE P., and LARDY, HENRY A. Phosphoric esters of biological importance. III. The synthesis of propanediol phosphate . . . . .	697
TOTTON, EZRA L., and LARDY, HENRY A. Phosphoric esters of biological importance. IV. The synthesis and biological activity of D-tagatose-6-phosphate . . . . .	701
JOHNSON, MARVIN J. A rapid micromethod for estimation of non-volatile organic matter . . . . .	707
SOODAK, MORRIS, PIRCIO, ANTHONY, and CERECEDO, LEOPOLD R. A colorimetric method for the estimation of uracil and cytosine . . . . .	713
DEKKER, CHARLES A., STONE, DAVID, and FRUTON, JOSEPH S. A peptide from a marine alga . . . . .	719
HENDERSON, L. M., RAMASARMA, G. B., and JOHNSON, B. CONNOR. Quinolinic acid metabolism. IV. Urinary excretion by man and other mammals as affected by the ingestion of tryptophan . . . . .	731
PARDEE, ARTHUR B., and POTTER, VAN R. Factors affecting the maintenance of oxidative phosphorylation in a kidney homogenate system . . . . .	739
JONES, MARY ELLEN, KOCH, F. C., HEATH, ARTHUR E., and MUNSON, PAUL L. Isolation of $\alpha$ -monopalmitin from hog pancreas . . . . .	755
KERR, STANLEY E., SERAIDARIAN, KRIKOR, and WARGON, MARIA. Studies on ribonucleic acid. II. Methods of analysis . . . . .	761
KERR, STANLEY E., SERAIDARIAN, KRIKOR, and WARGON, MARIA. Studies on ribonucleic acid. III. On the composition of the ribonucleic acid of beef pancreas, with notes on the action of ribonuclease . . . . .	773
GREENBERG, G. ROBERT. Inhibitory effect of muscle adenylic acid on anaerobic glycolysis of brain . . . . .	781
SNOKE, JOHN E., and NEURATH, HANS. Structural requirements of specific substrates for carboxypeptidase . . . . .	789

MASON, HOWARD S. The chemistry of melanin. VI. Mechanism of the oxidation of catechol by tyrosinase . . . . .	803
GILMORE, RICHARD C., JR., and SAMUELS, LEO T. The effect of previous diet on the metabolic activity of the isolated rat diaphragm . . . . .	813
GRUNERT, R. R., and PHILLIPS, PAUL H. Sodium and its relation to alloxan diabetes and glutathione . . . . .	821
CRANDALL, DANA I., and GURIN, SAMUEL. Studies of acetoacetate formation with labeled carbon. I. Experiments with pyruvate, acetate, and fatty acids in washed liver homogenates . . . . .	829
CRANDALL, DANA I., BRADY, ROSCOE O., and GURIN, SAMUEL. Studies of acetoacetate formation with labeled carbon. II. The conversion of $\gamma$ -(C <sub>7</sub> )-labeled octanoate to acetoacetate . . . . .	845
MAVER, MARY E., and GRECO, ANTOINETTE E. The hydrolysis of nucleoproteins by cathepsins from calf thymus . . . . .	853
MAVER, MARY E., and GRECO, ANTOINETTE E. The nuclease activities of cathepsin preparations from calf spleen and thymus . . . . .	861
SAUBERLICH, H. E., and BAUMANN, C. A. Further studies on the factor required by <i>Leuconostoc citrovorum</i> 8081 . . . . .	871
TOMARELLI, RUDOLPH M., NORRIS, R. F., GYÖRGY, PAUL, HASSINEN, J. B., and BERNHART, F. W. The nutrition of variants of <i>Lactobacillus bifidus</i> . . . . .	879
DVONCH, WILLIAM, and WHISTLER, ROY L. Water-soluble polysaccharides of sweet corn . . . . .	889
DINNING, JAMES S., and DAY, PAUL L. Creatinuria during recovery from aminopterin-induced folic acid deficiency in the monkey . . . . .	897
TOLBERT, N. E., CLAGETT, C. O., and BURRIS, R. H. Products of the oxidation of glycolic acid and <i>l</i> -lactic acid by enzymes from tobacco leaves . . . . .	905
DIETRICH, L. S., NICHOL, C. A., MONSON, W. J., and ELVEHJEM, C. A. Observations on the interrelation of vitamin B <sub>12</sub> , folic acid, and vitamin C in the chick . . . . .	915
INDEX TO VOLUME 181 . . . . .	921





# INFLUENCE OF FRUCTOSE AND OTHER CARBOHYDRATES ON THE NIACIN REQUIREMENT OF THE RAT

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(Received for publication, April 18, 1949)

Krehl *et al.* (1) have shown that certain types of carbohydrates alter the growth of rats on niacin-deficient rations. Starch, dextrin, glucose, and maltose increased growth, whereas sucrose decreased it, indicating that these carbohydrates modified the niacin requirement of the rat. These results might be explained by assuming that the type of carbohydrate influenced the amount of niacin synthesized by the intestinal flora. The evidence for this view was presented and discussed by these investigators (1). There are, however, many other possibilities which might explain these differences.

The experiments to be reported here were designed to test the possibility that varying amounts of niacin may be required in the metabolism of different sugar units.

## EXPERIMENTAL

The basal diet consisted of casein ("vitamin-free") 9, gelatin (U. S. P.) 3, L-cystine 0.15, carbohydrate 81, corn oil 3, and salts 4 (2). Vitamins were incorporated in the diet in the following amounts: thiamine 0.2, riboflavin 0.3, pyridoxine 0.25, calcium pantothenate 2, choline 100, inositol 10, 2-methylnaphthoquinone 0.1, biotin 0.01, and pteroylglutamic acid 0.1 mg. per cent. 2000 and 400 U. S. P. units of vitamins A and D respectively were given as a supplement to each rat twice weekly and 3 mg. of  $\alpha$ -tocopherol per rat were given once each week. Weanling rats develop a niacin deficiency in 7 to 14 days if sucrose is used in this ration.

The various experimental diets were made by substituting in the basal diet the following carbohydrates: levulose, 97 per cent practical grade or, in most instances, c.p. quality (Pfanstiehl); glucose, c.p. anhydrous (Merck); sucrose, commercial granulated; lactose, c.p. (Pfanstiehl); D-galactose (Pfanstiehl); white dextrin, N. F. V (Merck); corn-starch, (Argo); and, maltose (Nutritional Biochemicals). The starch, dextrin, sucrose, levulose, and glucose were free of detectable amounts (3) of niacin. The other carbohydrates were not assayed. Weanling male rats of National Institutes of Health (Sprague-Dawley) or Osborne and Mendel strains, weighing 40 to 50 gm., were maintained on the various diets in

individual suspended wire mesh cages. Litter mates were distributed to the various experimental groups by age and weight. Individual body weights were recorded three times weekly.

The niacin content of liver and thigh muscles was obtained by using the entire liver and the entire thigh muscles of one leg. The assay was made according to the microbiological method of Snell and Wright (3), slightly modified. Urinary *N*<sup>1</sup>-methylnicotinamide determinations were made as described by Huff and Perlzweig (4).

TABLE I  
*Growth of Rats As Influenced by Various Carbohydrates and Niacin*

No. of rats	Carbohydrate	Growth* per wk. per rat	
		No niacin added	2 mg. per cent niacin added
		gm.	gm.
10	Fructose	1.5 ± 0.7†	12.0
10	Sucrose	3.3 ± 0.94	17.2
5	½ glucose, ½ fructose	6.8 ± 1.1	22.0
15	Glucose	9.4 ± 0.71	23.0
5	Dextrin	13.5 ± 1.2	21.6
10	Starch	12.9 ± 1.04	22.7
5	Maltose	10.2 ± 1.27	28.6
5	½ glucose, ½ galactose‡	18.0 ± 1.14	19.3
5	½ " ½ lactose§	2.3 ± 1.17	12.0

\* The growth periods were 4 weeks on the unsupplemented diet and an additional week on the niacin-supplemented diet, except for the last two groups in which 3 weeks growth on the unsupplemented diet was used.

† Probable error of the mean result.

‡ All of the rats on this diet developed bilateral cataracts.

§ Lactose and galactose, alone, were also used in these experiments.

### Results

*Effect of Various Carbohydrates on Growth*—When diets containing the various carbohydrates were fed, wide differences in growth were observed (Table I). Each of the carbohydrates, except the glucose-galactose mixture, produced a deficiency of niacin as judged by growth of the same animals before and after niacin was added to the diet. The marked sparing action of the glucose-galactose mixture has not been described previously, nor has it been studied further here.

The most severe deficiencies were produced by fructose and sucrose. Glucose, a glucose-fructose mixture, and maltose produced somewhat less severe deficiencies. In other experiments (see Fig. 1 and Table II) glucose produced growth somewhat superior to that shown in Table I. Starch and

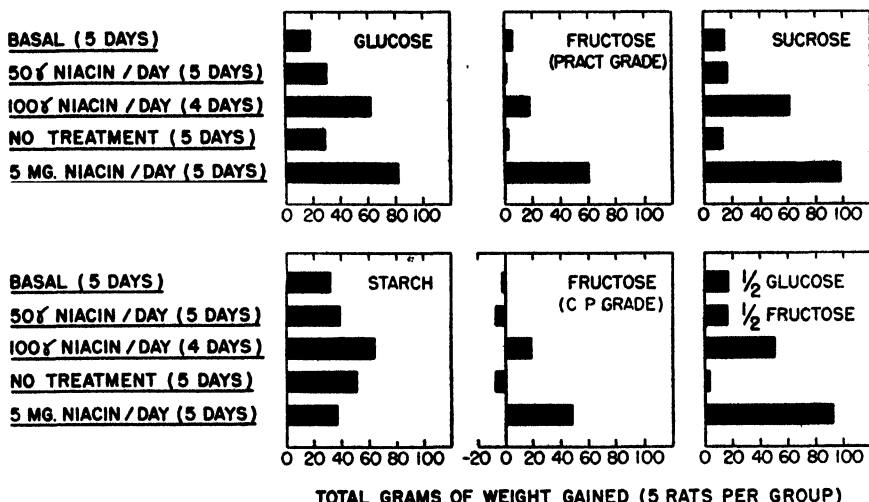


FIG. 1. Growth on various carbohydrates and test doses of niacin given subcutaneously. The growth periods represented above were begun after the rats had been on their respective diets for 3 weeks. The basal 5 day period, however, is a representative sample of their growth during the preceding 3 weeks.

TABLE II  
*Amount of Dietary Niacin Required for Maximal Growth*

Niacin  <i>mg. per cent</i>	Fructose			Sucrose		
	No. of rats	Mean	Probable error	No. of rats	Mean	Probable error
		<i>gm. per wk. per rat</i>			<i>gm. per wk. per rat</i>	
0	14	2.4	±0.59	14	3.1	±0.39
0.5	10	4.5	±0.52	10	6.6	±0.63
1.0	13	10.2	±0.75	19	13.3	±0.42
1.5	9	12.8	±0.94	10	15.4	±0.60
2.0	9	12.8	±0.62	10	15.2	±0.81
2.5	9	11.8	±0.56			
3.0				10	15.4	±0.63
10.0	14	12.6	±0.66	10	14.0	±0.74
	Glucose			Starch		
0	10	13.4	±0.57	14	14.1	±0.81
0.5	10	15.8	±0.93	15	17.1	±0.90
1.0	10	18.4	±0.69	10	16.4	±0.80
1.5	10	17.0	±1.00	5	18.6	±1.46
2.0	10	17.8	±0.76	10	16.3	±1.0
3.0	9	18.3	±0.95	10	17.6	±1.14

Growth periods 4 weeks in all groups.

dextrin gave relatively good growth and seemed to produce a rather mild deficiency. Even here, however, there was still a definite requirement for niacin as judged by the response to added niacin. A glucose-lactose mixture seemed to produce a severe deficiency, but the results were complicated by the toxic effects of the high lactose concentration. The toxic effect of high lactose concentrations in low fat diets is well known (5). The results reported here with sucrose, glucose, dextrin, and starch confirm those of Krehl *et al.* (1). The influence of fructose in increasing niacin requirements has not been reported previously.

It is of interest to compare the growth performance of the rats on sucrose and on a mixture of glucose and fructose which simulated sucrose. The rats on sucrose gained 3.3 gm. per week, while those on the glucose-fructose mixture gained 6.8 gm. per week, a difference of doubtful statistical significance. In another experiment (Fig. 1) the rats gained almost equally (4.6 and 4.3 gm. per week) on these two diets. In both experiments the rats were markedly deficient in niacin as judged by their growth response to it. The data would seem to exclude the possibility that a failure of the sucrose inversion mechanism is an important factor in these animals.

*Amount of Niacin Necessary to Produce Growth*—It seemed quite possible that the fructose content of sucrose was the factor responsible for the action of the latter in increasing the niacin requirement. This possibility was investigated by comparing the minimal amount of niacin necessary to produce maximal growth with fructose, sucrose, glucose, and starch (Table II). It can be seen that the growth peak occurred with 1.5 mg. per cent of added niacin in the fructose and sucrose diets. 1 mg. per cent of niacin gave maximal growth with glucose, while only 0.5 mg. per cent was necessary for starch.

The differences between growth in the absence of niacin are strikingly brought out in Table II. In this series of animals, glucose gave about 4 times as much growth as sucrose and about 5 times as much as fructose. It should also be noted that the maximal growth potential on these diets is different with the various sugars. Glucose and starch showed a considerably higher growth maximum than either sucrose or fructose. It is evident therefore that factors other than niacin limit the efficiency of utilization of fructose *in this type of diet*. Using another type of diet containing adequate protein, Bachmann *et al.* (6) observed no difference in growth rate between fructose and glucose.

The high niacin requirement with fructose and sucrose might be explained by assuming an increased metabolic need for niacin. Some evidence might be obtained relative to these possibilities by determining the relative tissue concentrations of niacin in these various groups of rats.

*Niacin Content of Liver and Muscles*—After 4 weeks on the various diets

most of the rats reported in Table II were sacrificed and the niacin content of the liver and hamstring muscles was determined (Table III). Rats sub-

TABLE III  
*Amount of Niacin in Tissues As Influenced by Amount of Dietary Niacin and Various Carbohydrates*

Niacin in diet <i>mg. per cent</i>	Fructose			Sucrose		
	No. of rats	Niacin		No. of rats	Niacin	
		Liver	Muscle		Liver	Muscle
		<i>γ per gm.</i>	<i>γ per gm.</i>		<i>γ per gm.</i>	<i>γ per gm.</i>
0	10	144 (131-150)	35 (33-38)	14	125 (112-140)	45 (32-55)
0.5	5	138 (125-155)	42 (35-45)	10	114 (100-120)	35 (25-44)
1.0	10	151 (131-180)	42 (38-47)	15	135 (110-155)	37 (25-50)
1.5	5	140 (134-150)	55 (47-60)	10	150 (130-200)	72 (54-96)
2.0	5	161 (152-180)	67 (60-70)	10	151 (130-163)	73 (55-79)
2.5	5	159 (130-190)	79 (75-85)			
3.0	5	172 (160-195)	78 (75-85)	10	185 (164-220)	96 (68-120)
10.0	5	187 (158-215)	75 (71-85)	5	170 (160-180)	69 (65-80)
	Glucose			Starch		
0	10	133 (118-150)	36 (32-40)	14	131 (120-150)	35 (30-49)
0.5	10	146 (124-180)	77 (69-90)	15	138 (106-175)	39 (33-48)
1.0	10	155 (142-190)	60 (52-66)	10	148 (132-183)	64 (55-67)
1.5	10	146 (130-162)	81 (50-120)	5	113 (103-120)	64 (62-67)
2.0	10	155 (130-180)	74 (47-95)	10	142 (130-150)	71 (68-80)
3.0	9	150 (140-165)	75 (60-82)	10	139 (130-150)	74 (65-85)

The values in parentheses indicate the range.

sisting on the niacin-free diets showed low tissue niacin, irrespective of the carbohydrate in the diet. These levels were comparable to those obtained previously in niacin-deficient rats (7) and to those reported by Singal *et al.* (8).

The results obtained from the liver analyses were somewhat difficult to interpret. None of the levels of niacin fed produced "normal" (*i.e.* about 180  $\gamma$  per gm.) liver concentration in the glucose and starch groups, nor was there a well defined maximum when these various groups were compared. On the other hand the fructose and sucrose groups showed approximately normal levels of liver niacin when 3.0 mg. per cent of dietary niacin was present.

The reason for the failure of the rats receiving glucose and starch to attain normal niacin levels in the liver is not known. It may have been related to the presence of fatty liver. Fatty liver seemed to be more prominent in rats receiving glucose or starch. Careful quantitative, comparative observations were not made, however. Krehl *et al.* (9) found that fatty liver may interfere with the storage of niacin in this organ.

The results were somewhat more consistent in the muscle assays. If one arbitrarily adopts 60  $\gamma$  per gm. as the lower limit of normal, then 2.0 and 1.5 mg. per cent of dietary niacin produced normal levels with fructose and sucrose respectively, while only 0.5 and 1.0 mg per cent were necessary for glucose and starch respectively.

One further point can be made if the data in Table III are compared with the growth data in Table II. Since the niacin levels in tissue were similarly depressed in all of the niacin-free diets, it is evident that rats can grow at a rate of 12 to 15 gm. per week in the presence of these low niacin concentrations when metabolizing glucose or starch, but hardly at all when metabolizing fructose or sucrose. This difference is even more apparent in the group receiving 0.5 mg. per cent of niacin, since the rats receiving starch grew maximally with low tissue niacin levels, while those on fructose and sucrose grew only about 30 per cent of the maximum, with similar levels of tissue niacin. It would seem therefore that the concentration of tissue niacin must be more nearly normal before fructose and sucrose can be metabolized efficiently.

*Results with Subcutaneous Test Doses of Niacin*—One difficulty that was inherent in the type of experiment reported above was that, although the amounts of niacin added to the diet were exactly equivalent in the various groups, the food intake varied considerably. This produced a constant and comparable intake of niacin per gm. of carbohydrate, but the total daily niacin intake varied with the quantity of diet consumed. It seemed clear that the principal factor influencing food intake in these rats was the amount of added niacin. Nevertheless such factors as food texture and palatability, which have been reported to influence food intake in rats (10), might have introduced some error.

Accordingly, another experiment was designed with fixed doses of niacin administered subcutaneously. Six matched groups of five rats each were

placed on diets without niacin, as indicated in Fig. 1. The weanling rats were maintained on these diets for 3 weeks. At the end of this period the total growth of each group of five rats in the following 5 days was recorded (basal 5 days). Then 50  $\gamma$  of niacin were given subcutaneously to each rat daily for 5 days. This was not sufficient to cause a growth response in the sucrose, glucose-fructose mixture, or the fructose groups, but did cause a response in the starch and glucose groups. The amount of niacin was then increased to 100  $\gamma$  daily. This produced a definite, though submaximal, response in all groups. The response of the fructose groups was much less than that of the others. At the end of this period niacin was discontinued and all growth rates declined. The groups on glucose and starch continued to grow above the basal level, however, while the other groups, especially those on fructose, promptly declined below the basal level. This would indicate that 100  $\gamma$  of niacin per day were sufficient to permit some storage

TABLE IV  
*Excretion of N<sup>1</sup>-Methylnicotinamide As Influenced by Dietary Niacin and Certain Carbohydrates*

Four rats in each group.

Niacin mg. per cent	Per 100 gm. of rat per day		
	Sucrose	Fructose	Starch
	$\gamma$	$\gamma$	$\gamma$
0	38	38	30
0.5	20	42	35
1.0	31	40	64
10.0	361	420	420

of niacin in the rats receiving dextrose and starch but not in the other groups.

It should be emphasized that the doses of niacin used here were given irrespective of body weight. The average body weights at the time represented in Fig. 1 were 51 and 41 gm. per rat in the fructose groups, 61 and 60 gm. in the sucrose and glucose-fructose groups, as compared with 92 and 66 gm. for the starch and glucose groups. Consequently, the rats in the fructose groups receiving 50  $\gamma$  of niacin actually received about twice the dose of niacin per unit of body weight as did those in the starch group, although the latter responded to this dose, while the former did not.

*Excretion of N<sup>1</sup>-Methylnicotinamide*—It is known that rats will excrete appreciable quantities of most vitamins only when they have a slight excess over their metabolic needs. It seemed possible therefore that additional information might be acquired by determining the level of niacin necessary to produce an increased excretion of N<sup>1</sup>-methylnicotinamide (the principal



derivative of niacin in the urine of rats (11)). Preliminary experiments indicated that 2.0 to 3.0 mg. per cent of dietary niacin were necessary to produce appreciable excretion in rats on sucrose diets, whereas less was required with starch diets.

Accordingly, small groups of rats were placed on various diets, as indicated in Table IV. Rats receiving none and 0.5 mg. per cent of added niacin showed similarly low excretion in all groups. However, 1.0 mg. per cent of dietary niacin produced an appreciable rise in excretion in the starch group but no comparable rise in the fructose and sucrose groups. 10 mg. per cent of added niacin produced a marked rise in excretion in all groups, as expected.

These data on *N*<sup>1</sup>-methylnicotinamide have limited statistical significance because of the small number of animals and determinations. They were not extended further since the results agreed with data previously obtained.

#### DISCUSSION

The data presented here seem to establish the fact that a relatively large amount of dietary niacin is required when fructose is the sole dietary carbohydrate. It appears fairly certain that the high niacin requirement with sucrose is due principally to its fructose content. Also it is probable from the studies reported by Krehl *et al.* (1) and from the data reported here that glucose in any of its forms results in a lower requirement for niacin.

The mechanism by which these differences in niacin requirement arise is not clear, however. Since fructose and sucrose supported less rapid growth than glucose when fed in the type of diet used in this study, it is possible that these sugars have a "toxic" effect in high concentrations, just as lactose and galactose have. Whether this would alter the requirement for niacin is not known.

It is believed that the evidence presented in this communication supports the idea that more niacin is actually needed in the metabolism of fructose, since rats receiving fructose grew well only when the concentration of tissue niacin approached normal, while rats receiving glucose grew well in the presence of reduced niacin levels. Furthermore, the data depicted in Fig. 1 indicate that more niacin is required to promote growth in niacin-deficient rats receiving fructose than in those receiving glucose.

From what is known concerning the pathway of enzymatic utilization of glucose and fructose, one would not expect any difference in niacin requirements. Nevertheless, many differences in the way the body handles these two sugars are known. Their absorption from the intestine is different (12). Their effects on the respiratory quotient and blood lactic acid are different (13, 14). Less deposition of body fat occurs in animals receiving fructose compared with glucose (6). The existence of separate

gluco- and fructokinases has been demonstrated (15). Many other differences are known, but which of these, if any, are responsible for the altered niacin requirement is not evident.

An influence of fructose on the niacin requirement would seem rather unusual. No other instance has been reported, to the best of our knowledge, in which the type of sugar unit metabolized increased the requirement for a vitamin.

#### SUMMARY

The niacin requirement of rats was greater on diets containing fructose or sucrose and less on diets containing dextrin or starch than on a diet in which the carbohydrate was glucose.

Rats using fructose grew well only when the levels of tissue niacin approached normal. Similar rats metabolizing glucose grew well in the presence of decreased concentrations of tissue niacin.

The data suggest the possibility that more niacin is consumed in the metabolism of fructose than of glucose.

Possible mechanisms by which dietary carbohydrates might alter the niacin requirement are mentioned.

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# ANALYSIS OF CERTAIN COMPONENTS OF SKELETAL MUSCLE DURING VITAMIN E DEFICIENCY\*

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Within the past few years, the varied effects of vitamin E deficiency were reviewed by Pappenheimer (1), Mason (2), Hickman and Harris (3), and Mattill (4). Further studies of the profound changes which its absence produces in the structure, composition, and particularly in the functional activity of muscle tissue have not yet led to an understanding of its rôle. The biochemical approach has been from two points of view which are not mutually exclusive. Tocopherol may inhibit oxidation *in vivo* as it does *in vitro*; or it may modify, possibly even participate directly in, cellular reactions, especially in those concerned with oxidation.

The antioxygenic aspect is represented by the sparing action of tocopherol on vitamin A and carotene which has been observed repeatedly, by the increased efficiency imparted to suboptimal doses of essential fatty acids (5), and the greater stability of the body fats when tocopherol is present in the diet (6). Furthermore, in its absence, peroxides have been demonstrated in the tissues (7); the brown pigment which appears in certain organs and tissues is believed to consist of polymerized substances originating in unusual products of oxidation (8, 9).

The relation of tocopherol to cellular oxidation is suggested by the higher rate of oxygen consumption by muscle strips from animals on vitamin E-deficient diets. Slices demonstrate this increase less markedly (10), and homogenates not at all (11). Tocopherol phosphate reduces the activity of the succinic dehydrogenase system in preparations from normal and dystrophic tissues alike (12). Its action may be direct or indirect; by precipitating calcium below the level of concentration necessary to activate diphosphopyridine nucleotidase, it would preserve diphosphopyridine nucleotide (DPN) which inhibits succinic dehydrogenase (13), or it may inhibit DPNase directly (14, 15), thus protecting DPN, as has been shown to happen in the lactic acid dehydrogenase system in heart muscle (16).

\* The content of this and the following paper was presented in part at the International Conference on Vitamin E, held under the auspices of the New York Academy of Sciences, April 15 and 16, 1949.

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In either case, the supply of DPN would be reduced in vitamin E deficiency.

Perhaps one of the functions of DPN is the transfer of phosphate (17). A diminished rate of phosphorylation of creatine by homogenates of muscles from certain species when deprived of vitamin E has been demonstrated (18); but normal phosphorylation was not restored by the presence of tocopherol phosphate.

The nature of the substrate undergoing increased combustion in dystrophic muscles is not known but the diminished creatine content of such muscles and the creatinuria, together with a considerable increase of creatine in the liver of dystrophic animals (19), tend to confirm the view that vitamin E may be linked with the metabolism of protein (8).

This paper records further attempts to explore the rôle of vitamin E as a biological antioxidant and as an agent in the regulation of cellular oxidation.

#### EXPERIMENTAL

*Biotin*—The observation that biotin is destroyed by autoxidizing fats and is protected by  $\alpha$ -tocopherol (20) prompted a study of the biotin content of normal and dystrophic muscles. On diets containing subnormal amounts of biotin, dystrophy due to lack of vitamin E might appear earlier than otherwise.

Hamsters, guinea pigs, and rabbits were placed on the dystrophy-producing diet heretofore used (21). Control animals on the same diet were given  $\alpha$ -tocopherol acetate<sup>1</sup> in olive oil by mouth, the guinea pigs and rabbits 15 mg. twice weekly, and the hamsters 7.5 mg.; the guinea pigs were also given 15 mg. of ascorbic acid every 3rd day.

Biotin, both free and combined, was determined microbiologically<sup>2</sup> by the method of Coryell *et al.* (22), turbidity after 24 hours being measured in a Coleman No. 11 spectrophotometer at 650  $m\mu$ .

Immediately after the animals were stunned and bled, distilled water homogenates were prepared as 1:20 dilutions. Tendinous material was removed by filtering through cheese-cloth, and aliquots were acidified, autoclaved, adjusted to pH 4.5, diluted to volume, and filtered. Because of the high content of sodium sulfate in the total biotin samples, a like amount was added to the standard biotin solution.<sup>3</sup>

The results of the biotin study (Table I) were disappointing in that the differences between control and dystrophic animals were not significant.

<sup>1</sup> Kindly supplied by Hoffman-La Roche, Inc., Nutley, New Jersey.

<sup>2</sup> For the original culture of *Lactobacillus arabinosus*, we are indebted to Dr. John R. Porter and the Department of Bacteriology.

<sup>3</sup> Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

In the muscles of hamsters on biotin-low diet, free biotin was considerably reduced, total biotin only slightly. Reduction of the biotin intake by inclusion of egg albumin in the diet did not affect the rapidity with which dystrophy appeared. If fatty acid peroxides are present in dystrophic animals, they do not destroy biotin *in vivo*, perhaps because of lack of contact or because biotin is combined with stabilizing substances.

TABLE I

*Biotin Content of Muscle from Animals with and without Vitamin E Supplement*

The values are given in micrograms per gm.  $\times 10^4$ . \*

	Total biotin		Free biotin	
	+ vitamin E	- vitamin E	+ vitamin E	- vitamin E
Hamsters (5, 5)*	37	32	8.6	7.7
" (5, 5) (low biotin)	30	27	5.3	4.1
Rabbits (7, 6)	12	13	4.5	3.0
Guinea pigs (3, 2)	17	28	5.0	6.4

\* Number of animals.

TABLE II

*QO<sub>2</sub> of Muscle Strips from Animals with and without Vitamin E Supplement*

	Ringer *	Ringer + glucose	Ringer + glucose + DPN
Guinea pigs, - vitamin E (5)*	2.0	2.0	2.4
" " + " " (5)	1.5	1.7	1.4
Rabbits, - vitamin E (7)	1.9	1.9	1.9
" + " " (5)	1.3	1.2	1.0
Hamsters, - vitamin E (5) (but no dystrophy)		3.1	3.6
" + " " (4)		2.3	2.4

\* Number of animals.

*Oxygen Uptake and DPN*—The increased rate of oxygen consumption by dystrophic muscle strips was again confirmed in the case of all three species (Table II). This was most pronounced in rabbit muscle. It was also evident in hamsters which showed no external signs of dystrophy, although they had been maintained on the deficient diet for a longer time than is usually necessary to produce it. Age was not a factor, since all the hamsters were within a few days of the same age.

The addition of various freely diffusible substances to the nutrient medium produced insignificant changes in the oxygen uptake, which implies that, even in dystrophic muscles, adequate amounts of substrate are

present. Any influence which DPN may have cannot be revealed merely by adding it to the medium in which the muscle is respiring, perhaps because it does not penetrate.

*Glutamine*—If there is an abnormal oxidation of muscle protein in vitamin E deficiency, this might be reflected in the level of free amino acids in muscle tissue. An amino aciduria has been demonstrated in patients with progressive muscular dystrophy (23). Since skeletal muscle contains a large proportion of its free amino acids as glutamine (24), changes in the level of this storehouse of labile amino groups might be a sensitive index to an altered oxidative deamination.

Glutamine was determined by the method of Hamilton (25), which depends on the fact that heating glutamine produces pyrrolidonecarboxylic acid which no longer reacts with ninhydrin to release carbon dioxide.

TABLE III  
*Glutamine in Muscle Carboxyl N from Animals with and without Vitamin E Supplement*

	Non-glutamine	Glutamine
	mg. per 100 gm.	mg. per 100 gm.
Guinea pigs, + vitamin E (6)*. . .	21.7 $\pm$ 8.2	4.8 $\pm$ 2.7
" " - " " (7) . . .	20.8 $\pm$ 4.1	1.5 $\pm$ 1.35
Rabbits, + vitamin E (5). . . .	26.7 $\pm$ 4.2	8.7 $\pm$ 2.2
" - " " (5) . . . .	27.6 $\pm$ 4.4	6.7 $\pm$ 1.5

\* Number of animals.

From 2 to 4 gm. of muscle tissue were homogenized with appropriate volumes of picric acid, and the CO<sub>2</sub> from the amino acids present was measured in the Van Slyke manometric apparatus. Some of the samples of muscle were stored at -40° before analysis.

As shown in Table III, the skeletal muscle of dystrophic guinea pigs showed a striking decrease in glutamine content; in rabbits, the decrease from normal was less marked, and perhaps not significant. This species difference may be due to the fact that on the vitamin E-deficient diet rabbits become dystrophic within 2 to 4 weeks, whereas guinea pigs require 6 to 7 weeks.

The total non-glutamine amino acid content of the muscles was influenced little, if at all, by vitamin E deficiency. A study of the distribution of other amino acids in this condition might be revealing.

The function of glutamine appears to be different from that of glutamic acid (26). Glutamine serves as a neutral storage (27) and transport (28) form of labile amino groups. Its synthesis is an endothermic reaction (29) for which respiration or glycolysis (30) supplies the necessary energy.

Homogenates of rat and guinea pig livers synthesize glutamine if adenosine triphosphate,  $Mg^{++}$ , and ammonia are present (31); with pigeon liver preparations (32) and with an enzyme system obtained from sheep brain (33), liberation of inorganic phosphate parallels the reaction.

The observed reduction in glutamine in the muscles of dystrophic animals may well be an immediate and direct result of the loss, through combustion, of energy that is normally stored in high energy phosphate bonds. A significant decrease in the glutamine level of muscle has also been demonstrated in guinea pigs deficient in ascorbic acid (34). Whether the function of vitamin E is specific and how it contributes to the normal economy of muscle metabolism in different species remain to be determined.

The author is indebted to Dr. George Kalnitsky and Dr. H. A. Mattill for their suggestions.

#### SUMMARY

In nutritional muscular dystrophy resulting from a deficiency in vitamin E, the free and total biotin content of skeletal muscle from hamsters, rabbits, and guinea pigs did not vary from that of control animals. When the biotin content of the diet was lowered (hamsters), that of the muscles was also decreased, irrespective of the adequacy of the vitamin E intake. It is therefore unlikely that vitamin E acts as an antioxidant in tissue to preserve biotin, although this relationship has been observed in the autoxidative destruction of biotin by unsaturated fats *in vitro*.

The heightened consumption of oxygen by dystrophic muscle from guinea pigs and rabbits is again confirmed; muscle from hamsters that had not yet developed dystrophy showed an increased uptake. The addition of various substrates and of DPN to the nutrient medium produced no alteration in the utilization of oxygen.

The glutamine content of skeletal muscles of dystrophic guinea pigs was about one-third that of muscle from control animals, but the average content of non-glutamine amino acids was unchanged. In vitamin E-deficient rabbits, the difference was less marked.

The possible significance of the decreased glutamine content is briefly discussed with reference to the energy relationships. Whether and how vitamin E participates directly or indirectly in normal muscle economy remain to be determined.

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## TRANSAMINATION IN THE MUSCLES OF ANIMALS DEPRIVED OF VITAMIN E\*

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Since the dicarboxylic acid system holds a central position in tissue metabolism, the study of possible alterations in this system in the muscles of vitamin E-deficient animals was continued. Transamination between aspartic and  $\alpha$ -ketoglutaric acids was investigated because of the many functions which it appears to perform in carbohydrate, fat, and particularly protein metabolism, and in the regulation of cellular respiration. Transamination first claimed attention through the work of Braunstein and Kritsman (1). The limitations and specificity of transaminases were revealed by Cohen (2), the two known transaminases were isolated in reasonable purity by Green, Leloir, and Nocito (3), and the conclusion of several investigators that pyridoxal phosphate is the coenzyme has been widely confirmed.

### EXPERIMENTAL

The dietary production of muscular dystrophy has been described (4). Control rabbits and guinea pigs, on the same deficient diet, were given orally 15 mg. of  $\alpha$ -tocopherol acetate<sup>1</sup> in olive oil twice a week, and guinea pigs also received 10 mg. of ascorbic acid three times per week. The animals were killed by a blow on the head and portions of the muscles of the hind legs were cooled, trimmed free of connective tissue and fat, quickly weighed on a torsion balance, minced with scissors, and homogenized in distilled water. After being strained through cheese-cloth to remove shreds of connective tissue, the mince (1:10 for guinea pigs, 1:5 for rabbits) was pipetted into chilled Warburg flasks.

The manometric method used for the determination of transamination was essentially that of Green, Leloir, and Nocito (3), as adapted by Ames and Elvehjem (5) to the measurement of transaminase activity in tissue homogenates. However, the control flasks, instead of lacking  $\alpha$ -ketoglutaric acid, contained the complete reaction mixture; the carbon dioxide

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<sup>1</sup> Kindly supplied by Hoffman-La Roche, Inc., Nutley, New Jersey.

released at zero time represented that coming from tissue and reagents prior to transamination.

To provide further criteria for determining the significance of the results, several modifications were applied. (a) Aniline citrate was replaced in the side arm of some flasks by 0.50 ml. of 1 N hydrochloric acid for the purpose of measuring the carbon dioxide formed through the spontaneous decomposition of oxalacetic acid to pyruvic acid during the 10 minute reaction period. (b) In certain experiments, 0.05 mg. of pyridoxal phosphate (as the calcium salt)<sup>2</sup> in aqueous solution was substituted for 0.50 ml. of water. (c) To ascertain whether any apparent change in the rate of transamination in dystrophic muscle might be due to an increased removal, by some alternative pathway, of the oxalacetic acid formed, solutions of sodium oxalacetate in various concentrations, instead of sodium aspartate and sodium  $\alpha$ -ketoglutarate, were used as substrates for various concentrations of tissue. The volume of the reaction mixture was maintained at 3.0 ml.

The rate of transamination is expressed as microliters of CO<sub>2</sub> per 100 mg., wet weight, of tissue, and also per 10 mg., dry weight, of homogenate, and per mg. of total nitrogen of homogenate. The dry weight of samples was determined by evaporation to constant weight at 110°. Total nitrogen was determined by a micro-Kjeldahl method. In the experiments on decomposition of oxalacetic acid, the oxalacetic acid recovered is reported as per cent of the amount originally added.

#### RESULTS AND DISCUSSION

As shown in Table I, the transaminase activity of homogenates of dystrophic guinea pig muscle was less than half that of normal muscle; dystrophic rabbit muscle showed a similar but less striking diminution. These results are uniform, whether expressed on the basis of wet weight of the tissue, of dry weight, or of total nitrogen content of the homogenate. The altered transaminase activity is thus not a reflection of the gross changes in tissue structure and composition accompanying dystrophy. Age was not a significant factor, for the control animals were, as nearly as possible, of the same age as the experimental animals at the time of use. A few determinations made on older animals suggest that transaminase activity, like succinic dehydrogenase activity (6), decreases with age.

In both species, the addition of 0.05 mg. of pyridoxal phosphate (Table I) produced no change in the evolution of CO<sub>2</sub> from either homogenate. This amount of pyridoxal phosphate was such as to allow coenzyme-enzyme complex formation in the 12 minutes of the equilibration period even in the presence of the aspartic acid substrate (7), and could therefore

<sup>2</sup> Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

be expected to restore the enzyme activity if coenzyme concentration had been the limiting factor (8).

An apparent decrease in transamination might be occasioned by an increased rate of removal of oxalacetate by other reactions (9). Table II shows that a 1:20 homogenate of dystrophic guinea pig muscle allowed recovery of approximately the same per cent of oxalacetic acid after the reaction period as did normal muscle. With a 1:10 homogenate of dystrophic muscle, the recovery of added oxalacetate was the same as with

TABLE I  
*Transaminase in Muscle of Guinea Pigs and Rabbits*

	Average amount of CO <sub>2</sub> evolved		
	Per 100 mg., wet weight	Per 10 mg., dry weight	Per mg. N
Guinea pigs			
	$\mu$ l.	$\mu$ l.	$\mu$ l.
Normal (11)*.....	505 $\pm$ 117	289 $\pm$ 83	242 $\pm$ 53
Dystrophic (10) .....	231 $\pm$ 91	136 $\pm$ 36	123 $\pm$ 36
Normal (5).....	470		
" + pyridoxal phosphate†.....	468		
Dystrophic (4) .....	194		
" + pyridoxal phosphate† ..	182		
Rabbits			
Normal (10)....	92 $\pm$ 14	84 $\pm$ 16	63 $\pm$ 15
Dystrophic (8) .....	70 $\pm$ 15	51 $\pm$ 13	40 $\pm$ 12
Normal (3).....	90		
" + pyridoxal phosphate†.....	86		
Dystrophic (3) .....	65		
" + pyridoxal phosphate†... ..	60		

\* Number of animals.

† 0.05 mg. of pyridoxal phosphate in 0.5 ml. of water.

the 1:20 homogenate, whereas with normal muscle it was, if anything, less, a result exactly opposite to that which would indirectly account for the diminished transamination.

The data on rabbits further justify the conclusion that the reduction in transamination is not an artifact produced by the increased removal of oxalacetic acid by some alternative pathway. In the case of the two higher concentrations of rabbit muscle homogenate, the recovery of oxalacetic acid was noticeably greater with dystrophic muscle than with normal muscle, indicating that normal muscle possesses a better mechanism for disposing of excess oxalacetate than does dystrophic muscle.

Incidentally, the amount of carbon dioxide released by hydrochloric acid at the end of the 10 minute reaction period was in all cases found to be small, about 10 per cent of the total CO<sub>2</sub> released by aniline citrate. No significant amount of oxalacetic acid was therefore broken down to pyruvic acid and carbon dioxide in either normal or dystrophic muscle homogenates.

In a study of the transamination reaction between alanine and  $\alpha$ -ketoglutaric acids, Vyshepan found no difference between normal and dystrophic rabbit muscle (10). There seems to be no quantitative information on transamination between aspartic and  $\alpha$ -ketoglutaric acids in rabbit skeletal muscle, but, according to Cohen and Hekhuis (9) this reaction in rat skeletal muscle is about 20 times the magnitude of the alanine- $\alpha$ -keto-

TABLE II  
*Decomposition of Oxalacetic Acid by Muscle Homogenates*

Oxalacetic acid added	Amount and concentration of homogenate	Per cent recovery of added oxalacetic acid	
		Normal	Dystrophic
Guinea pigs			
mg.	ml.		
0.8	0.5 (1:20)	86 (1)*	85 (3)
1.6	0.5 (1:20)	84 (6)	86 (3)
1.6	1.0 (1:10)	80 (3)	88 (3)
Rabbits			
0.8	0.5 (1:20)	89 (2)	89 (4)
1.6	0.5 (1:5)	74 (4)	83 (3)
1.6	1.0 (1:5)	58 (4)	70 (3)

\* Number of animals in the average.

glutaric exchange. Analogies are uncertain because of species variations, but Vyshepan's system might not have revealed any differences existing on so much smaller a scale.

The effects of a decrease in transamination on metabolism as a whole can only be conjectured. Koshtoyants and Ryabinovskaya (11) found that in embryonic rabbit muscle the onset of transamination is coincident with the appearance and accumulation of phosphocreatine and the establishment of rapid contraction.

Glutamine does not undergo transamination (12). Dystrophic muscles contain less glutamine than normal muscles (13). The blocking of amidation could lead to a higher concentration of glutamic acid, and, if this cannot undergo the normal amount of transamination, its disposal might be channeled to oxidative deamination. Loss of enzymes due to partial inanition (14) might account in part for the changes observed in dystrophy.

The authors are indebted to Dr. George Kalnitsky for his suggestions and continued interest.

#### SUMMARY

The aspartic-glutamic transaminase activity of skeletal muscle homogenates from guinea pigs and rabbits, made dystrophic by lack of vitamin E, was one-half to two-thirds that found for muscle homogenates from control animals. Activity was not restored by addition of the coenzyme, pyridoxal phosphate.

The decrease was not the result of increased removal of oxalacetate by some alternative pathway; excess oxalacetate was more readily disposed of by normal than by dystrophic muscle homogenates.

The possible relations between diminished transamination and the increased oxygen consumption of dystrophic muscle are briefly discussed.

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# THE RATES OF ABSORPTION AND THE FORMATION OF LIVER GLYCOGEN BY METHIONINE, CYSTINE, AND CYSTEINE\*

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Chase and Lewis (2) found the rate of absorption of DL-methionine from the gastrointestinal tract of the white rat to be 53 mg. per 100 gm. of body weight per hour.<sup>1</sup> This value was based upon the increase in the amino nitrogen content of the gastrointestinal tract after feeding DL-methionine determined by the Van Slyke method. Andrews, Johnston, and Andrews (3) reported that methionine was absorbed more rapidly than cystine in a dog with an isolated intestinal loop. These investigators determined total sulfur, by the Benedict procedure, in the washings of the unabsorbed material in the loop. Both of these observations were made some time ago and, apparently, are the only ones in the literature.

The rate of absorption of L-cystine from the gastrointestinal tract of the white rat was determined by Wilson (4) to be 30.5 mg. per hour. Wilson estimated the amount of unabsorbed cystine by the Folin-Marenzi (5) cystine method. Sullivan and Hess (6) repeated the experiment of Wilson and obtained the same rate of absorption when the cystine was estimated by the Folin-Marenzi method, but when the Sullivan cystine method was used the rate of absorption was found to be 49.3 mg. per hour. The Okuda (7) iodometric cystine method was also used by Sullivan and Hess (6) and it gave a rate of 49.2 mg. per hour. The lower rate given by the Folin-Marenzi method was believed to be due to the presence of material other than cystine in the gastrointestinal tract that reacted as cystine in this method but did not react when the more specific Sullivan and Okuda methods were used.

Recently Schofield and Lewis (8) have studied the rates of absorption of the various optical isomers of alanine and found some differences in their rates of absorption. The early work on methionine was done with the DL isomer and on cystine with the L isomer. The present study is concerned with the rates of absorption of the several optical isomers of

\* A report on a part of this work was presented at the meeting of the American Society of Biological Chemists at Atlantic City, March, 1949 (1).

<sup>1</sup> Hereafter the expression "per 100 gm. of body weight per hour" will be abbreviated to "per hour."



these two amino acids and of cysteine. The absorption rates, following administration of the amino acids for various periods, have been determined by estimation of the amounts of the amino acids remaining in the gastrointestinal tract by colorimetric methods and also by the determination of the total sulfur present. The rate of absorption of L-methionine did not differ significantly from that of D- or DL-methionine, nor were any significant differences found between the rates of absorption of L-, D-, or DL-cystine. The rate of absorption of mesocystine, however, was lower than the rates of the other cystine isomers.

Chase and Lewis (2) found that the feeding of DL-methionine did not lead to the formation of liver glycogen, a finding confirmed in the present work. Vars (9) has reported that the injection of methionine into a phlorhizinized dog results in the secretion of extra urinary glucose. The formation of glycogen in the liver following the administration of L-cystine according to Butts, Blunden, and Dunn (10) and of either L-cystine or L-cysteine according to Medes and Vitanza (11) does not occur, although Dakin (12) had early reported extra glucose formation in the phlorhizinized dog following L-cystine injection. Stöhr (13), however, did find an increase in liver glycogen after the ingestion of both L-cystine and L-cysteine, and Kubo (14) reported an increase in blood sugar in rabbits after feeding L-cystine. The experiments described in this paper show that both L-cystine and L-cysteine caused an increase in liver glycogen.

#### EXPERIMENTAL

The general procedure was the same as that previously used by Sullivan and Hess (6). Male white rats, weighing 150 to 200 gm., previously fasted for 24 hours, were used. The amino acids were administered, by stomach tube, as the sodium salts, except in one experiment in which cysteine hydrochloride was employed. The amount of each amino acid fed was approximately 75 mg. per 100 gm. of body weight for the 1 hour absorption period, and 150 and 250 mg. per 100 gm. for the 2 and 3 hour absorption periods respectively. The entire gastrointestinal tract was removed at the end of the absorption period and extracted, in a Waring blender, with 10 per cent aqueous sulfosalicylic acid and then filtered. In those instances in which the total sulfur was determined, 10 per cent trichloroacetic acid replaced the sulfosalicylic acid.

Cystine was determined as previously described by Sullivan and Hess (6). Methionine was determined by the McCarthy-Sullivan (15) method as used by Hess and Sullivan (16). Glutathione was determined by the Okuda (7) iodometric method. Total sulfur was estimated by the method of Pollack and Partansky (17).

The L-cystine and synthetic DL-methionine were Eastman products.

Resolution of the DL-methionine was effected by the procedure of Teas, Horowitz, and Fling (18). For the preparation of the DL-cystine and the mesocystine and the resolution of the DL-cystine the methods of du Vigneaud, Dorfman, and Loring (19) were used. The rotations of the various isomers were measured in a Schmidt and Haensch polarimeter and were found to be within the ranges reported for D- and L-cystine and D- and L-methionine. The cysteine hydrochloride was prepared by the reduction of the L-cystine with tin and hydrochloric acid, removal of the tin, and crystallization of the hydrochloride. The purity of the several isomers of cystine was tested by the Sullivan method with a sample of L-cystine of known purity as the standard. All of the isomers were at least  $99.8 \pm 0.3$  per cent pure. The purity of the cysteine hydrochloride was determined by an Okuda titration on an aliquot of known concentration. A second aliquot of the same cysteine solution was used for a cystine determination by the Sullivan method after the cysteine had been oxidized. The purity of the cysteine hydrochloride was at least 98.6 per cent. The L-, D-, and DL-methionine samples were matched against a standard sample of synthetic DL-methionine of known purity by the colorimetric method of McCarthy and Sullivan (15). All of the methionine samples were at least  $99.4 \pm 0.5$  per cent pure.

Glycogen was determined by the method of Good, Kramer, and Somogyi (20). In the experiments in which both the rate of absorption of the particular amino acid and the liver glycogen were determined, the rats were fasted for 48 instead of 24 hours. During the fasting period the rats had free access to water.

### Results

*Cystine and Cysteine*—The results for the various isomers of cystine and for cysteine are given in Table I. The rate of absorption of L-cystine previously found by Sullivan and Hess (6) was 49.3 mg. per hour, in close agreement with the present value of 49.9 mg. per hour. The rate of absorption of D-cystine is 45.6 mg. per hour, slightly less than that of L-cystine but not significantly different. Loring and du Vigneaud (21) found that the DL isomer is a racemic compound and not a racemic mixture. This fact may explain our finding that DL-cystine has a slightly higher rate of absorption than either the D or the L isomer. Mesocystine had an absorption rate of 41.3 mg. per hour, a value definitely lower than that of any other isomer. The rate of absorption of L-cysteine is lower than the rate of any of the cystine isomers, especially when the cysteine was used as the hydrochloride and not as the sodium salt. The rates of absorption, calculated from the amounts of total sulfur in the gastrointestinal tract, did not differ materially from those obtained by the Sulli-

TABLE I

*Rates of Absorption of Optical Isomers of Cystine and of Cysteine Fed As Sodium Salts*

Compound	No. of animals	Time	Rate, mg. per 100 gm. per hr.	
			Sullivan-Hess method	Total sulfur
		<i>hrs.</i>		
DL-Cystine	6	2	53.6	52.1 (4)*
	1	1	54.8	
	1	3	52.5	
Average.			53.6 $\pm$ 1.5†	52.1 (4)
L-Cystine	17	2	50.0	49.0 (4)
	1	1	44.6	41.0 (1)
	7	3	47.2	45.5 (4)
Average.			49.9 $\pm$ 2.8	47.5 (9)
D-Cystine	6	2	45.7	46.0 (4)
	1	1	43.4	43.0 (1)
	2	3	47.3	47.0 (2)
Average.			45.6 $\pm$ 2.6	45.7 (7)
Mesocystine	4	2	42.8	41.3 (4)
	1	1	38.8	39.7 (1)
	2	3	38.3	37.8 (2)
Average			41.3 $\pm$ 3.3	40.4 (7)
L-Cysteine	12	2	41.2	38.5 (4)
	1	1	39.0	36.6 (1)
	8	3	44.7	42.8 (4)
Average			41.4 $\pm$ 2.6	38.9 (9)
	2‡	2	25.7	23.4 (2)

\* The figures in parentheses are the number of rats used for the average.

† Standard deviation.

‡ The cysteine hydrochloride was not neutralized.

van colorimetric method. This finding confirms the previous conclusion of Sullivan and Hess (6) that the rate based on the cystine determination by the Folin-Marenzi method was too high.

*Methionine*—In Table II are given the results on the isomers of methionine. Both D- and DL-methionine gave values varying but little from

that of 35.7 mg. per hour for L-methionine. These values, however, are lower than those obtained for the sodium salt of DL-methionine by Chase and Lewis (2) who reported an absorption rate of 53 mg. per hour. These investigators used the Van Slyke amino nitrogen method to determine the amount of methionine remaining in the gastrointestinal tract. The

TABLE II  
*Rates of Absorption of Optical Isomers of Methionine Fed As Sodium Salts*

Compound	No. of animals	Time  <i>hrs.</i>	Rate, mg. per 100 gm. per hr.	
			Sullivan-McCarthy	Total sulfur
D-Methionine	6	2	35.2	32.8 (6)*
	1	1	37.2	38.8 (1)
	11	3	35.7	35.5 (4)
	Average . . . . .		35.6 $\pm$ 1.4†	35.8 (11)
L-Methionine	6	2	35.9	34.2 (6)
	1	1	33.0	
	4	3	37.1	35.1 (4)
	Average . . . . .		35.7 $\pm$ 1.1	34.5 (10)
DL-Methionine	6‡	2	34.1	
	7	2	38.7	38.4 (4)
	1	1	39.6	
	3	3	34.5	
	Average . . . . .		38.5 $\pm$ 2.5	38.4 (4)
	6‡	2	39.2	

\* The figures in parentheses are the number of rats used for the average.

† Standard deviation.

‡ The rate in this series was calculated from amino nitrogen determination.

amino nitrogen method of Pope and Stevens (22) has been found to give more satisfactory results than the Van Slyke procedure and one series of determinations by this method was run. Absorption rates of 34.1 mg. per hour for L-methionine and 39.2 mg. per hour for DL-methionine were found, in confirmation of the values obtained by the colorimetric method. The rates of absorption calculated from the amount of sulfur remaining in the gastrointestinal tract were essentially the same as those obtained by both the colorimetric methionine method and the estimation of the amino nitrogen.

**Glycogen**—The data on the determination of glycogen in the livers of the rats fed L-cystine, L-cysteine, and L-methionine are given in Table III. The glycogen content of the livers of the rats fed L-methionine for 2 and 3 hours was not increased over that of the controls. The feeding of both L-cystine and L-cysteine increased the glycogen content of the livers. After 2 hours the L-cystine was more effective in increasing liver glycogen than the L-cysteine, owing, perhaps, to its more rapid rate of absorption; after 3 hours the L-cysteine produced more glycogen than did the L-cystine. These results confirm the findings of Stöhr (13), who also fed the sodium salts of the amino acids, and found, after 6 hours, liver glycogen contents of 0.37 and 0.58 per cent from cystine and cysteine respectively. Medes and Vitanza (11), following the procedure of Butts *et al.* (10), suspended the insoluble cystine in gum tragacanth, thus, perhaps, reducing its absorbability. The control rats of Medes and Vitanza,

TABLE III  
*Glycogen Content of Livers of Rats Fed Amino Acids*

Compound	No. of rats	Time <i>hrs.</i>	Glycogen
			<i>per cent</i>
Controls	13		0.04 $\pm$ 0.015
L-Cysteine	11	2	0.83
	6	3	1.11
L-Cystine	8	2	1.54
	7	3	0.46
L-Methionine	4	2	0.04
	4	3	0.03

fasted for 24 hours, had a high glycogen content, averaging 0.114 per cent, compared with 0.04 per cent glycogen in our control rats, while their rats fed the amino acids were fasted for 36 hours. In one experiment with cystine in which the procedure of Stöhr was followed Medes and Vitanza found a slight increase in liver glycogen.

**Cystine and Methionine in Liver**—The livers from several of the rats fed L-cystine and L-methionine and also from four control rats were deproteinized with 10 per cent trichloroacetic acid. The livers from the cystine-fed rats showed an increased glutathione content, 0.35 per cent as compared with 0.18 per cent in the control livers. Sullivan and Hess (6) had previously made the same observation. The free cystine content of the livers, determined by the Sullivan method applied to the trichloroacetic acid filtrates, of both sets of rats was of the same order, approximately 5 mg. per cent. No free methionine was found by the McCarthy-

Sullivan (15) method in the trichloroacetic acid filtrates from the livers of the control rats, while those fed L-methionine for 2 hours showed an average of 0.11 per cent methionine. The glutathione content of the livers of the methionine-fed rats was the same as that of the livers of the control rats. Determination of the total sulfur in the protein-free extracts of the livers of the rats fed L-methionine showed a marked increase over that of the control rats. The sulfur content of the free methionine found in these extracts accounted for 20 per cent of the extra sulfur. The nature of the remaining sulfur is now under study. A portion of it may be homocystine and there is also the possibility that cystathionine may be present, although so far there is no evidence of the latter compound.

#### SUMMARY

The sodium salts of L-, DL-, D-, and mesocystine were fed to white rats for periods of 1, 2, and 3 hours. The rates of absorption were calculated by determining the cystine remaining in the gastrointestinal tract. The values for L-, D-, and DL-cystine were 49.9, 45.6, and 53.6 mg. per 100 gm. per hour respectively, not significantly different, while the rate for mesocystine was 41.3 mg. per 100 gm. per hour. The above values were based on the determination of cystine by the Sullivan method. Estimation of the cystine as calculated from the total sulfur content of the gastrointestinal tract gave results in close agreement. No differences in the rates of absorption were found after 1, 2, or 3 hours of feeding. Cystine hydrochloride was absorbed slowly, while cysteine, fed as the sodium salt, was absorbed at a decidedly faster rate, 25.7 mg. and 41.4 mg. per hour respectively.

The rates of absorption of D-, L-, and DL-methionine, fed as the sodium salts, were 35.6, 35.7, and 38.2 mg. per 100 gm. per hour respectively. These values are based upon the determination of methionine by the McCarthy-Sullivan colorimetric method. The methionine was also determined by calculation from the total sulfur and the amino nitrogen of the gastrointestinal tract. The values thus obtained were in agreement with those determined by the colorimetric method.

The feeding of both L-cystine and L-cysteine produced extra liver glycogen, while methionine did not.

The feeding of L-cystine produced extra glutathione in the liver; methionine did not. Considerable unexplained extra sulfur was found in the livers of the methionine-fed rats.

All of the glycogen determinations were run by Mrs. B. T. Smith, whose assistance is gratefully acknowledged.

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# SOME ASPECTS OF THE METABOLISM OF HYDROXYPROLINE, STUDIED WITH THE AID OF ISOTOPIC NITROGEN\*

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Previous studies *in vivo*, in which isotopically labeled amino acids were fed to mice or rats, have shown that intact animals can convert ornithine into arginine, glutamic acid, and proline (1, 2), and proline into hydroxyproline, ornithine, and glutamic acid (3). The problem of the metabolic interconversions of these structurally similar 5-carbon amino acids has now been further studied with the aid of hydroxyproline labeled with N<sup>15</sup>.

Hydroxyproline containing 29.5 atom per cent excess N<sup>15</sup> has been synthesized by a slight modification of the method of Leuchs, Giua, and Brewster (4) and has been fractionated into its two racemic forms. Only the hydroxy-DL-proline which contains the naturally occurring hydroxy-L-proline was fed.<sup>1</sup> 0.519 gm. of the N<sup>15</sup> hydroxy-DL-proline was added to the stock diet and fed to three adult male rats for 3 days, during which time the urine and feces were collected. The rats were then killed and a number of amino acids isolated from the total body proteins. The isotope analyses of the body constituents and of the urine fractions are given in Table I.

The largest part of the N<sup>15</sup> fed as hydroxy-DL-proline was excreted, a small amount in the feces and a relatively large amount in the urine. The urinary urea and NH<sub>3</sub> had high and approximately equal concentrations of N<sup>15</sup>, but the isotope concentration of the total urinary nitrogen was still higher, indicating that some of the dietary hydroxyproline was probably excreted as such. Analyses of the urine indicated that, during the hydroxyproline feeding, about 10 mg. of  $\alpha$ -amino acid N were excreted in excess of that excreted in a corresponding control period with the same stock diet. This is equivalent to 94 mg. of hydroxyproline. The quantities involved were too small to permit isolation of hydroxyproline from the urine in this case.

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<sup>1</sup> The revised nomenclature for the isomers of hydroxyproline is used in this paper (5). Hydroxy-DL-proline in this system corresponds to the *dl*( $\alpha$ )-hydroxyproline of Leuchs *et al.* (4), while allohydroxy-DL-proline refers to the *dl*( $\beta$ ) racemate of Leuchs.



An appreciable amount of the  $N^{15}$  fed as hydroxyproline was incorporated into the body proteins, but its distribution in the various constituents was in striking contrast to the results obtained when other  $N^{15}$ -labeled amino acids were fed. In similar experiments, with varying diets and amounts of added isotopic amino acids, it has been found that leucine, lysine, glycine, serine, proline, and histidine, when fed, replaced between 6 and 30 per cent

TABLE I

*Concentration of Isotopic Nitrogen after Feeding Isotopic Hydroxy-DL-proline to Rats*

A total of 0.519 gm. of hydroxy-DL-proline, containing 16.3 mg. of  $N^{15}$ , was fed to two rats for 3 days.

Sample analyzed		$N^{15}$ con- centration*	$N^{15}$ recovered
		atom per cent	mg
Excreta	Hydroxy-DL-proline fed	100	
	Urine, total N	1.87	6.8
	“ urea	1.11	
	“ $NH_3$	1.11	
Body constituents	Feces	0.65	0.5
	Total protein N	0.13	5.0
	“ non-protein N	0.31	2.0
	Amide N	0.15	
Amino acids from total proteins	Hydroxyproline	0.075	
	Proline	0.051	
	“ after “washing out”	0.048	
	Tyrosine	0.078	
	Arginine	0.108	
	Ornithine from arginine	0.047	
	Amidine N “ “	0.204	
	Glutamic acid	0.27	
	Aspartic “	0.18	
Total recovery .			14.3

$$* N^{15} \text{ concentration} = \frac{N^{15} \text{ atom } \% \text{ excess in sample isolated} \times 100}{N^{15} \text{ atom } \% \text{ excess in hydroxyproline fed (i.e., 29.5)}}$$

of the corresponding amino acid in the proteins of the rats in 3 to 4 days (6-10). When L-proline was fed, it was calculated that a minimum of 30 per cent of all the proline in the organs and 7 per cent of all the remaining carcass proline was replaced by dietary proline in 3 days (3). In the present experiment, the hydroxyproline isolated from the bodies of the rats fed  $N^{15}$ -hydroxyproline contained only a trace of isotopic nitrogen. Less than 0.1 per cent of the hydroxyproline of these rats had been replaced by dietary hydroxyproline in 3 days. In fact, under the not strictly com-

parable conditions of our experiments, there was appreciably more  $N^{15}$  in the hydroxyproline of the rat's body after proline than after hydroxyproline feeding. These results indicate that most of the hydroxyproline occurring in the body proteins is probably derived from proline that is already bound, presumably in peptide linkage, and not from free amino acid hydroxyproline. This finding is analogous to the observations of Brown, Roll, Plentl, and Cavalieri (11) who report that when adenine containing  $N^{15}$  was fed appreciable amounts of  $N^{15}$  were found both in the adenine and the guanine of the nucleic acids, whereas when  $N^{15}$ -labeled guanine was fed, there was isotope in neither the adenine nor guanine isolated.

In the present experiment, more  $N^{15}$  was found in the glutamic acid than in the hydroxyproline from the rats, a further indication of the poor incorporation of dietary hydroxyproline. In other feeding experiments with  $N^{15}$ -labeled amino acids, the glutamic acid isolated has always been relatively rich in isotope, owing to its high metabolic activity and its participation in transamination reactions, but it has been poorer in isotope than the isolated amino acid corresponding to the compound fed or those derived more or less directly from it. Here the glutamic acid, aspartic acid, arginine, and tyrosine have probably picked up  $N^{15}$  from  $NH_3$  or other degradation products of the dietary  $N^{15}$ -hydroxyproline. The greater part of the isotope of the arginine was in the amidine, or urea precursor, portion of the molecule.

The proline isolated from the rat proteins contained even less  $N^{15}$  than did the isolated hydroxyproline. It is possible that all of this isotope came indirectly from breakdown products of the hydroxyproline fed. The possibility that a small amount of the body proline arises from direct reduction of dietary hydroxyproline cannot be categorically ruled out on our evidence, but this conversion, if it occurs at all, is quantitatively an unimportant reaction.

Dietary hydroxyproline and dietary proline are handled by the body in different ways. These findings are in accord with a number of observations in which the metabolism and the oxidation rates of proline and hydroxyproline *in vitro* have been shown to differ (12-17). Of especial interest in this regard are the recent nutrition studies of Womack and Rose (17), showing that proline but not hydroxyproline can replace a part of the arginine requirement of growing rats and that hydroxyproline, when fed in relatively large amounts, may even inhibit growth.

Hydroxyproline occurs in appreciable amounts in only a few proteins, notably in gelatin (18, 19), and presumably collagen, where it comprises about 13 per cent of the total amino acids, and elastin where it occurs to the extent of 2 per cent (20). It seems to be completely lacking in casein,

globin, serum globulin and albumin, and animal muscle and kidney protein (19, 21). It is, however, certainly a normal constituent of some animal proteins, especially in connective tissues, and not an artifact of isolation. This may be inferred from the finding of widely differing isotope concentrations in proline and hydroxyproline isolated from the same animal source (3).

#### EXPERIMENTAL

*Synthesis of 4-Hydroxyproline with  $N^{15}$* — $\delta$ -Chloro- $\gamma$ -valerolactone- $\alpha$ -carboxylic ethyl ester, prepared from epichlorohydrin and sodium malonic ester according to Traube and Lehmann (22), was chlorinated and converted to  $\alpha, \delta$ -dichlorovalerolactone by the method of Leuchs, Giua, and Brewster (4). The method of conversion of the latter compound into hydroxyproline was modified so as to permit economical use of the isotopic nitrogen.

$NH_3^*$ , generated by adding a solution of 17.2 gm. of  $NH_4NO_3^*$  (31.5 atom per cent  $N^{15}$ , Eastman Kodak) dropwise to a hot concentrated NaOH solution, was aerated into an ice-cold aqueous alcoholic solution of 8.5 gm. of  $\alpha, \delta$ -dichlorovalerolactone. The reaction was allowed to proceed at room temperature for 10 days, after which time the remaining free  $NH_3$  was recovered by aeration of the heated solution into acid. To recover the  $NH_3$  from the ammonium salt, the solution was cooled, made alkaline with  $Ba(OH)_2$ , and once again heated and the  $NH_3$  aerated into HCl. Barium sulfate and chloride ions were quantitatively removed, the solution was treated with charcoal, and the filtrate boiled for several hours with CuO. The filtered solution was evaporated to a small volume and successive crops of copper hydroxyproline crystals were obtained on repeated refrigeration and evaporation.

This method of fractional crystallization has been shown (4) to separate the DL compound containing the naturally occurring hydroxy-L-proline from the DL-allo isomer. The first two crops, consisting of 0.965 gm. of Cu salt, were well formed, dark blue crystals, typical of the less soluble hydroxy-DL-proline copper salt tetrahydrate. 0.802 gm., dried for 6 hours at  $110^\circ$  over  $P_2O_5$  *in vacuo*, lost 146 mg. or 18.2 per cent. Theory for  $C_5H_8O_3N)_2 \cdot 4H_2O$ , 18.2 per cent  $H_2O$ .

The copper salt was decomposed with  $H_2S$  and the hydroxy-DL-proline recrystallized from water-alcohol.

$C_5H_8O_3N$ . Calculated, N 10.7; found, N 10.8; 29.5 atom %  $N^{15}$  excess

*Feeding of Hydroxy-DL-proline*—Two adult male rats having a combined weight of 440 gm. were maintained on a diet consisting of 15 per cent casein,

68 per cent corn-starch, 5 per cent yeast, 4 per cent Osborne and Mendel salt mixture (23), 2 per cent cod liver oil, and 6 per cent refined cottonseed oil (Wesson oil). After a preliminary period of 5 days, isotopic hydroxy-DL-proline (29.5 atom per cent  $N^{15}$  excess) was added to the diet for 3 days. The rats completely consumed 90 gm. of stock diet, containing 0.519 gm. (0.66 mm per rat per day) of the isotopic hydroxyproline, without any spillage, and maintained constant weight. The urine and feces were collected as nearly quantitatively as possible during the 3 days. At the end of the 3rd day, the rats were killed with ether and the gastrointestinal tracts removed and discarded.

*Excreta*—The combined urine contained 134 mg. of ammonia N with 0.327 atom per cent  $N^{15}$  and 984 mg. of urea N. Urea, isolated as the dioxanthanol derivative (24), was found to contain 0.328 atom per cent  $N^{15}$ . The fecal N, 0.26 mg., contained 0.193 atom per cent  $N^{15}$ . The urine contained about 10 mg. of amino acid nitrogen over the amount excreted in a control period on the same diet, as determined by the method of Hamilton and Van Slyke<sup>2</sup> (25). The urine was observed to have a deeper orange-red color than is normal. This color became deeper as the urine stood in the refrigerator and interfered with observation of the specific rotation of the urine.

*Body Constituents*—The entire bodies of the rats, exclusive of the gastrointestinal tracts, were minced and combined. The non-protein nitrogen was separated by extraction with 6 per cent trichloroacetic acid. There were 2.12 gm. of non-protein N of 0.092 atom per cent  $N^{15}$  and 13.06 gm. of protein N of 0.038 atom per cent  $N^{15}$ . A sample of amide nitrogen, obtained by aeration of the protein hydrolysate after the addition of  $Ba(OH)_2$ , contained 0.045 atom per cent  $N^{15}$ .

From the total hydrolyzed proteins a number of amino acids were isolated by methods previously referred to (3). Tyrosine (0.023 atom per cent  $N^{15}$ ) was isolated by isoelectric precipitation, glutamic acid hydrochloride (0.080 atom per cent  $N^{15}$ ) and copper aspartate (0.053 atom per cent  $N^{15}$ ) from their precipitated barium salts, arginine hydrochloride (0.032 atom per cent  $N^{15}$ ) by way of the flavanate and proline (0.015 atom per cent  $N^{15}$ ), and hydroxyproline (0.022 atom per cent  $N^{15}$ ) by the method of Bergmann.

To minimize error due to possible contamination of isolated proline with isotopic hydroxyproline, the carcass proline was "washed out" by adding 46 mg. of non-isotopic hydroxyproline to a solution of 205 gm. of the isolated proline. Proline was reisolated as its  $CdCl_2$  double salt, which was found to contain 0.014 atom per cent  $N^{15}$ .

<sup>2</sup> The author is indebted to Dr. Halvor Christensen for this analysis and to Mr. Frank Rennie and Mrs. Eleanor Schroeder for carrying out the isotope analyses.

A sample of the isolated arginine hydrochloride was cleaved by heating in  $\text{Ba}(\text{OH})_2$  solution for 48 hours. The ammonia from the amidine part of the molecule contained 0.060 atom per cent  $\text{N}^{15}$ ; the ornithine contained 0.014 atom per cent  $\text{N}^{15}$ .

#### SUMMARY

4-Hydroxyproline was so synthesized as to contain 29.5 atom per cent excess  $\text{N}^{15}$ . This was fractionated into its two DL forms and some of the hydroxy-DL-proline, containing the natural hydroxy-L-proline, was added to the stock diet fed to two rats for 3 days.

The largest part of the isotope was recovered in the urine, the urea and  $\text{NH}_3$  having high concentrations.

Some of the isotope was found in the body proteins. The very low isotope concentration of the hydroxyproline isolated from the body proteins indicated that less than 0.1 per cent of the hydroxyproline in these rats had been derived from the dietary hydroxyproline in 3 days. A higher concentration of  $\text{N}^{15}$  was found in the glutamic acid, aspartic acid, and arginine of the proteins and probably came indirectly from degradation products of the hydroxyproline.

The body proline contained only traces of  $\text{N}^{15}$ , indicating that little if any of the proline of the body is derived from dietary hydroxyproline.

The hydroxyproline of the proteins is not derived to any appreciable extent from dietary hydroxyproline but rather from the oxidation of proline which is already bound, presumably in peptide linkage.

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# GLUTAMINE IN THE PRODUCTION OF TETANUS TOXIN\*

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A strain of *Clostridium tetani* under investigation in this laboratory produces high titers of exotoxin when cultivated on a medium containing trypsin-digested protein and high concentrations of iron salts (1). Toxin production fails when acid- or alkali-hydrolyzed protein (casein) is substituted for the enzyme digest, and it has been shown (2) that at least two acid-labile substances or groups of substances are required in order to obtain suitable yields. In addition to these, certain amino acids (tryptophan, tyrosine, phenylalanine, and cystine) are known to be required and others will undoubtedly prove necessary as the simplification of the medium proceeds. This phase of the investigation has been hampered by the fact that certain free amino acids exert an inhibitory effect on toxin production. This is particularly true of synthetic serine, in which instance the inhibition has been shown (3) to arise from the presence of the D form of the compound.

It has now been possible to show that glutamine is involved in growth and toxin formation. Glutamic acid, while it favors growth, has led to low yields of toxin. Although there appears to be relatively little free dicarboxylic amino acid in the tryptic digest of casein used in our experiments, both glutamic acid and aspartic acid are readily recognized after acid hydrolysis of almost every peptide-containing fraction which has been prepared from the material, by whatever method of separation. Presumably some of these peptides contain glutamine rather than glutamic acid, and in a form which is both relatively heat-stable and available to the tetanus organism. Much time may be required to isolate, characterize, and test individually such peptides; nor can suitably quantitative comparisons be made until other unknown factors can be supplied to the medium in purified form. It consequently seems desirable to report at this time the general fact that glutamine is involved, leaving for subsequent reports a further quantitative elaboration of the observation.

Glutamine has, of course, been shown to act as a growth essential or stimulant for a number of bacterial species (4, 5), although its functions in microbial growth are still largely matters of speculation (6, 7). There is also a possibility that glutamine enters into the streptogenin molecule

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to help make up an essential, vitamin-like grouping (8). A coenzyme-like relationship of glutamine to a glucose-fermenting enzyme of the tetanus cell is suggested by experiments being carried out by Lerner in this department, which are reported separately (9).

#### EXPERIMENTAL

Although the effect of glutamine in stimulating toxin production has been demonstrated in several types of control media, the simplest plan has been to lower the concentration of tryptic digest until the toxin yields

TABLE I  
*Effect of Glutamine on Tetanus Toxin Production*

Tube No.	Tryptic digest of casein*	Glutamine†	Toxin‡
1	300 mg. per 20 ml.		90
2-7	150 " " 20 "		35
			32
			32
			30
			30
			32
8-13	150 " " 20 "	5 mg. per 20 ml.	65
			40
			50
			55
			50
			50

\* A commercial preparation, N-Z-Case, Sheffield Farms, Inc., New York.

† Sterilized by Seitz filtration and added to the autoclaved medium.

‡ The figures denote flocculating units of toxin per ml.

are sharply depressed. Addition of glutamine then significantly increases the yield.

The medium, which consists essentially of a tryptic digest of casein, meat infusion, glucose, and inorganic salts, together with an excess of iron ("iron by hydrogen," Merck), has been fully described elsewhere, together with all the details of the procedure (2). For simplicity, Table I shows only the variables in a single type experiment.

The growth occurring in Tubes 8 to 13 was significantly greater than that in Tubes 2 to 7, but less than in Tube 1. It is clear that 5 mg. of glutamine do not fully replace 150 mg. of trypsinized casein (representing approximately 30 mg. of glutamic acid). Further increments of the amide added to the basal media thus far available have been without marked effect. The addition of asparagine has produced slight but irregu-

lar increases. It must remain for further experiment to define the other deficiencies in the medium and to make possible an elucidation of the quantitative aspects of the glutamine effect.

#### SUMMARY

Glutamine appears to replace, at least in part, certain glutamine-containing peptides occurring in a tryptic digest of casein which influence the production of tetanus toxin under defined conditions.

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# THE RÔLE OF GLUTAMINE IN THE GLUCOSE METABOLISM OF *CLOSTRIDIUM TETANI*\*

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In the course of an investigation of the glucose metabolism of a mutant strain of *Clostridium tetani* in this laboratory, it was found that the sugar was completely broken down to carbon dioxide and ethanol in the process of an essentially alcoholic fermentation (1). The glucose-fermenting ability of this strain was directly dependent upon the concentration of inorganic iron in the growth medium, in a straight line semilogarithmic proportion. Cells from media which had been made deficient in iron by means of calcium chloride precipitation at alkaline pH, or by extraction with 8-hydroxyquinoline, had very poor glucose-fermenting ability. It was postulated that an enzyme or coenzyme concerned in the breakdown of glucose was elaborated by cells grown in iron-rich medium and not by cells grown in iron-deficient medium.

An attempt has now been made to isolate and identify this postulated enzyme. The test system consisted of washed suspensions of *Clostridium tetani* which had been grown for 18 to 20 hours in iron-deficient medium (medium containing 0.03  $\gamma$  of iron per ml., or less), glucose as substrate, and phosphate buffer of pH 7.0, in an atmosphere of purified nitrogen in Warburg respirometers. Various preparations were tested for their ability to stimulate the production of carbon dioxide from glucose by this system.

Several methods of extraction of tetanus bacilli grown in high iron media yielded a stimulatory substance which was free from protein and apparently of small molecular weight. Further studies showed that the glucose-fermenting stimulatory activity could also be extracted from yeast, mammalian liver, and muscle. Concurrently with isolation procedures, known coenzymes and vitamins were tried for stimulatory activity without effect. Chromatographic separation of deproteinized liver extracts on columns of activated charcoal, and counter-current distribution in a phenol-water system, gave fractions that were active in amounts as small as 100  $\gamma$ .

Simultaneous studies by Mueller and Miller (2) on factors in the growth medium responsible for toxin production by this strain of *Clostridium*

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*tetani* showed that a fraction of tryptically digested casein was active in causing high yields of toxin by this microorganism. The fraction contained a peptide composed of glutamic acid, or glutamine, and other amino acids. A tryptic digest of casein and also the fraction, mentioned above, of trypsinized casein had a stimulatory effect on the glucose metabolism of iron-deficient cells similar to the effect of extracts of high iron cells, yeast, liver, or muscle; pure glutamine was then found to be exceptionally active.

When added to washed suspensions of iron-deficient cells, glutamine in quantities as small as 50  $\gamma$  caused an increase in their production of carbon dioxide from glucose, as measured in Warburg respirometers; increasing amounts of glutamine caused an increase in carbon dioxide production in semilogarithmic proportion, as shown below.

L-Glutamine added, mg . . . .	0	0.05	0.5	1.0	2.5
CO <sub>2</sub> evolved in 120 min., $\mu$ l..	57.1	77.2	112.6	128.0	170.1

TABLE I  
*Stimulation of Iron-Deficient Cells*

Fe suspensions	CO <sub>2</sub> per hr.	$Q_{CO_2}^{N_2}$	Stimulation
	$\mu$ l.		per cent
Low .....	20.8	4.3	
" + glutamine (1.5 mg.) .....	63.5	13.2	207
High.....	90.8	15.8	268

High iron cells (grown in the deficient medium described above, to which 1.0 mg. of reduced iron per ml. had been added) produced 2 to 7 times as much CO<sub>2</sub> as did low iron cells. The CO<sub>2</sub> production by suspensions of low iron cells with excess glutamine added approached that of high iron cells. The results of a comparative experiment are given in Table I.

Although the  $Q_{CO_2}$  of different cell suspensions varied somewhat, the stimulation of low iron cells by glutamine repeatedly approached the magnitude of CO<sub>2</sub> evolution by high iron cells.

Microbiological assay with *Lactobacillus arabinosus* (3) of some of the most active fractions of liver extracts separated by counter-current distribution showed that glutamic acid was present after hydrolysis. Asparagine had less than 8 per cent of the stimulatory activity of glutamine on a molar basis. It seems probable that the stimulatory effects noted with liver extract fractions and with trypsin digests of casein are due to the presence of glutamine or glutamine-containing peptides in both materials.

It is of interest to note that glutamine has a somewhat similar effect on the glycolytic activity of several strains of hemolytic streptococci, in that the addition of this compound has been found to stimulate the production of lactic acid from glucose by washed cell suspensions (4).

#### SUMMARY

1. Glutamine apparently plays an important rôle in glycolysis by *Clostridium tetani*. Cells from iron-deficient medium lack the ability to ferment glucose, in contrast to cells from iron-rich medium. Glutamine can induce this glycolytic activity in iron-deficient cells, as measured by CO<sub>2</sub> evolution.

2. Increasing amounts of glutamine cause an increasing degree of stimulation. Excess glutamine increases the  $Q_{CO_2}$  almost to the level of high iron cells.

3. It is suggested that the stimulatory activity noted in extracts of high iron cells, yeast, liver, etc., is due to the presence of glutamine, either free or in peptide linkage.

4. The rôle of glutamine in the intermediary metabolism of *Clostridium tetani* is being investigated further.

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# PUTRESCINE AND RELATED COMPOUNDS AS GROWTH FACTORS FOR *HEMOPHILUS PARAINFLUENZAE* 7901\*

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Early studies of the nutritional requirements of *Hemophilus parainfluenzae* resulted in identification of the diphosphopyridine and triphosphopyridine nucleotides as the previously undefined factor "V" required for growth of this organism (1, 2). Subsequently, *H. parainfluenzae* has been used frequently for the assay of coenzymes I and II (3-6). A peptone medium proved adequate in these studies, but efforts to grow *H. parainfluenzae* in a defined medium containing coenzyme I were unsuccessful (7). Our own experiments showed that growth of this organism failed in a defined medium which contained each of the presently known growth factors, but did occur when any of several natural materials was added. Efforts to identify the additional unidentified growth factor were therefore initiated.

The present report describes the isolation, identification, and specificity of putrescine as a growth factor for one strain of this organism. A preliminary report of these findings has previously appeared (8).

## EXPERIMENTAL

*Stock Culture*—*H. parainfluenzae*, ATCC 7901, was used in all the experiments. Stock cultures were maintained on slants of a supplemented North gelatin agar (Difco); prior to preparation of the slants each 100 ml. of sterile North agar (cooled to 45°) were supplemented with 2 ml. of a sterile yeast extract prepared in the following manner.

50 gm. of bakers' yeast were suspended in 25 ml. of distilled water and poured into 75 ml. of boiling water containing 3.4 gm. of  $\text{KH}_2\text{PO}_4$ . After holding at 80-85° for 20 minutes, the mixture was filtered with suction through a pad of filter aid (Hyflo Super-Cel) and sterilized by Seitz filtration.

Cultures were transferred weekly, incubated for 24 hours at 37°, and stored at room temperature.

*Basal Medium*—The composition of the medium used for the assay of

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source materials and in the isolation of putrescine is shown in Table I. This complex mixture was devised to prevent the possible occurrence of multiple deficiencies during the purification of the growth factor; many of its ingredients are not essential nutrients for *H. parainfluenzae*.

TABLE I  
*Composition of Basal Medium\**

Component	Amount per 5 ml. (double strength)	Component	Amount per 5 ml. (double strength)	Component	Amount per 5 ml. (double strength)
	mg.		mg.		
DL-Aspartic acid	10	L-Cystine	1	Biotin	0.01 $\gamma$
L-Glutamic "	10	L-Tyrosine	1	p-Aminobenzoic acid	0.01 "
DL-Alanine	10	Glycine	1	Folic acid	0.1 "
L-Arginine·HCl	2	Glucose	10	Inositol	200 "
L-Lysine·HCl	2	Sodium acetate	60	Coenzyme I	1 "
DL-Methionine	2		$\gamma$	MgSO <sub>4</sub> ·7H <sub>2</sub> O	1 mg
L-Leucine	1	Adenine sulfate	100	CaCl <sub>2</sub> ·2H <sub>2</sub> O	400 $\gamma$
DL-Threonine	2	Guanine·HCl	100	FeSO <sub>4</sub> ·7H <sub>2</sub> O	135 "
DL-Serine	2	Uracil	100	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	4 "
L-Proline	1	Thiamine chloride	1	CuSO <sub>4</sub> ·5H <sub>2</sub> O	4 "
DL-Tryptophan	2	Riboflavin	1	CoCl <sub>2</sub> ·6H <sub>2</sub> O	4 "
DL-Valine	2	Nicotinic acid	5	MnSO <sub>4</sub> ·H <sub>2</sub> O	3 "
DL-Phenylalanine	2	Nicotinamide	5	K <sub>2</sub> HPO <sub>4</sub>	15.6 mg.
L-Histidine	1	Pyridoxine·HCl	20	KH <sub>2</sub> PO <sub>4</sub>	1.4 "
DL-Isoleucine	2	Ca pantothenate	10		
		Choline chloride	50		

\* The amino acid mixture has been described (9) and was prepared by the same methods. 5 ml. quantities of double strength medium were distributed in 150 ml. Pyrex milk dilution bottles; test materials and distilled water were added to a final volume of 10 ml. The pH was adjusted to 7.8 with NaOH and the medium was sterilized by autoclaving for 15 minutes at 120°. The coenzyme I was sterilized by Seitz filtration and added to the cooled sterile medium. Following inoculation, cultures were incubated for 48 hours at 37°. Growth was then estimated turbidimetrically with the Evelyn colorimeter.

*Inoculum*—The inoculum medium contained Difco-proteose peptone (No. 3) 2 per cent, glucose 0.1 per cent, NaCl 0.2 per cent, K<sub>2</sub>HPO<sub>4</sub> 0.2 per cent, and Difco yeast extract 0.1 per cent. 10 ml. lots were placed in milk dilution bottles and autoclaved. Immediately prior to inoculation of the medium by transfer of growth from the agar slant stock cultures, 0.2 ml. of the Seitz-filtered extract of fresh yeast was added to 10 ml. of this sterile medium. After 10 to 12 hours of incubation at 37°, growth in the

medium was sufficiently heavy to allow preparation of the inoculum. The cells were removed by centrifugation, washed once with sterile, 0.9 per cent NaCl solution, then resuspended with sufficient sterile saline to permit 95 per cent transmission of incident light (Evelyn colorimeter, 660 m $\mu$  filter; 16 mm. tube; sterile saline = 100). 0.1 ml. of this suspension was added to each 10 ml. of assay medium (Table I).

When heavier inocula were used than that described above, the sensitivity of the assay was markedly reduced, the results were more erratic, and growth occasionally occurred in the blank tubes. It is necessary, therefore, to control carefully the size of the inoculum.

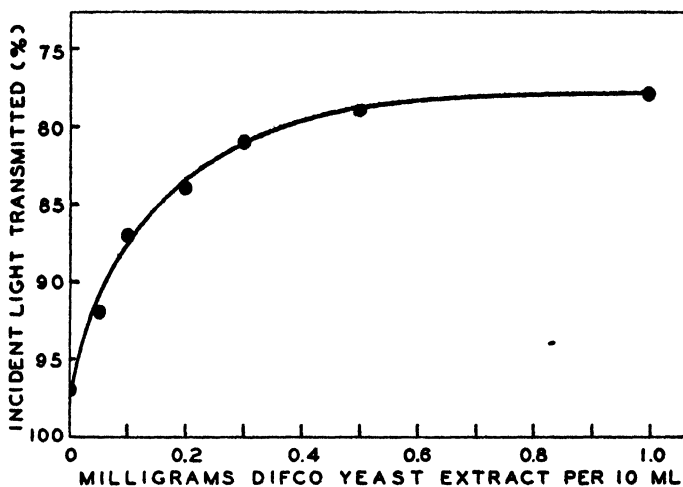


FIG. 1. The response of *H. parainfluenzae* to Difco yeast extract

### Results

The failure of the basal medium to support growth and the growth response to added Difco yeast extract are illustrated in Fig. 1. Numerous attempts to increase further the maximum yield of cells by supplementation to, or adjustment of, the nutrient mixture were unsuccessful.

The assay results on concentrates of the growth factor for *H. parainfluenzae* were expressed in terms of a standard of orange juice solids, since orange juice was used in the successful isolation of the growth factor.

*Distribution and Properties of Growth Factor*—Every crude material examined contained the growth factor. Difco yeast extract, corn steep liquor, and peas were comparatively rich source materials. Orange and grapefruit juices, either fresh or canned, were approximately a fifth as active, on the dry weight basis, as Difco yeast extract.

Preliminary isolation attempts were made with yeast extract. The

stimulatory substance in yeast was soluble in water but could not be extracted with ethanol (95 per cent), ether, or butanol. Continuous extractions of aqueous solutions of yeast extract with butanol at pH 1, 6.5, and 10 resulted in extraction of small amounts of active material at pH 10.0, but not at lower pH values. The active factor in yeast was not destroyed by autoclaving aqueous solutions at pH 1 to 10 for 1 hour, or by refluxing with 6 N HCl for 8 hours. The active substance was precipitated by phosphotungstic acid and mercury salts, but very little purification was achieved by the application of these procedures. Similarly, while the factor in yeast was quantitatively removed from aqueous solutions by the cation exchange resin, Amberlite IR-100,<sup>1</sup> and eluted from the resin with 3 N HCl, only a 4-fold concentration of the activity resulted from this procedure. The growth factor was completely inactivated by treating solutions of yeast extract with nitrous acid or with benzoyl chloride.

Since these properties indicated that the growth factor was a strongly basic amine, the choice of a source material providing a lower concentration of inactive basic constituents than yeast presented the possibility of the successful application of the same purification procedures previously found to be ineffective. This supposition was confirmed by subsequent experiments utilizing orange juice as the source material. Concurrent with these attempts at isolation of the growth factor, various naturally occurring, acid-stable amines were assayed for possible activity.

*Isolation of Putrescine from Orange Juice*—5 liters of canned, unsweetened orange juice (Monarch) were clarified by filtration through a pad of Hyflo Super-Cel. The filtrate (pH 3.8) was passed at a rate of 50 ml. per minute through a 3 cm. glass column containing 50 gm. of Amberlite IR-100. The column was then washed with 1 liter of distilled water. Only 13 per cent of the total activity was recovered in the effluent (Table II). The column was then washed with 5 liters of 2.4 N HCl and the HCl eluate, which contained the growth factor, was concentrated to near dryness *in vacuo*. A 40-fold concentration of the active substance was achieved by this single cycle of adsorption and elution (Table II).

At this point in the investigation, assay of pure organic amines showed that spermine and spermidine possessed growth-promoting activity, and since it appeared possible that these bases were the active growth factor, the procedures described by Rosenheim (10) for their isolation were next applied.

The active resin eluate was dissolved in 50 ml. of distilled water, 25 gm. of NaOH were added, and the mixture was distilled with steam. All of the activity was recovered in 3 liters of distillate. This distillate was adjusted to pH 6.8 with 10 per cent  $H_2PO_4$  and evaporated to 50 ml. *in*

<sup>1</sup> The Resinous Products and Chemical Company, Philadelphia 5, Pennsylvania.

*vacuo* in an attempt to isolate spermine as the insoluble phosphate (10). However, no spermine phosphate was recovered. Furthermore, spermidine phosphate did not precipitate when alcohol was added to the concentrated steam distillate (*cf.* Dudley (11)). The active compound in the distillate appeared not to be identical, therefore, with either spermine or spermidine. Addition of sodium picrate solution to this concentrated steam distillate resulted in the formation of a crystalline picrate (1.036

TABLE II

*Activity of Fractions from Orange Juice in Promoting Growth of H. parainfluenzae*

Fraction	Units of activity*	Units per mg. solids
Clarified canned orange juice.	447,500	1
Resin effluent....	58,000	0.1
“ wash water	0	0
“ HCl eluate .. .	220,000	40
Steam distillate . . .	240,000	470
Picric acid derivative . . .	220,000	210
Free base (calculated)	220,000	1300

\* 1 unit of activity is the growth response equivalent to that obtained with 1 mg. of orange juice solids.

TABLE III

*Identification of Putrescine as Active Compound from Orange Juice*

Derivative	Compound isolated	Putrescine
Picrate, °C.. . . .	M.p. 264	M.p. 263
Hydrochloride, % . . .	N 17.48, 17.43	N 17.39
Benzoyl, °C. . . . .	M.p. 178-180	M.p. 178-180
Phenyl isocyanate, °C. . .	“ “ 235-237	“ “ 235-237

gm.) which was highly active in stimulating growth of *H. parainfluenzae* (Table II).

Following several recrystallizations, the picrate was converted to the hydrochloride, and then to the benzoyl and phenyl isocyanate derivatives, following the general procedures of Dudley *et al.* (12). Preliminary identification of the isolated base as *putrescine* was confirmed by analysis, and by agreement of the melting points of the various derivatives with those found for the corresponding derivatives of an authentic sample of putrescine (Table III). Mixed melting points showed no depression. The isolated putrescine accounted for 50 per cent of the growth-promoting activity of the 5 liters of orange juice used in its isolation.

*Specificity of Requirement for Putrescine*—The results of assays of compounds structurally related to putrescine are summarized in Table IV. Only putrescine, spermidine, spermine, and agmatine showed activity.

TABLE IV  
*Specificity of Amine Requirement of H. parainfluenzae*

Active compounds	Inactive compounds
Putrescine	Ornithine
Spermidine	Ethylenediamine
Spermine	Trimethylenediamine
Agmatine	Cadaverine
	Hexamethylenediamine
	<i>n</i> -Butylamine
	1-Aminobutanol-4
	Tetramethyleneglycol
	Arcaine
	Pyrrolidine
	Triethylenetetramine
	Tetraethylenepentamine

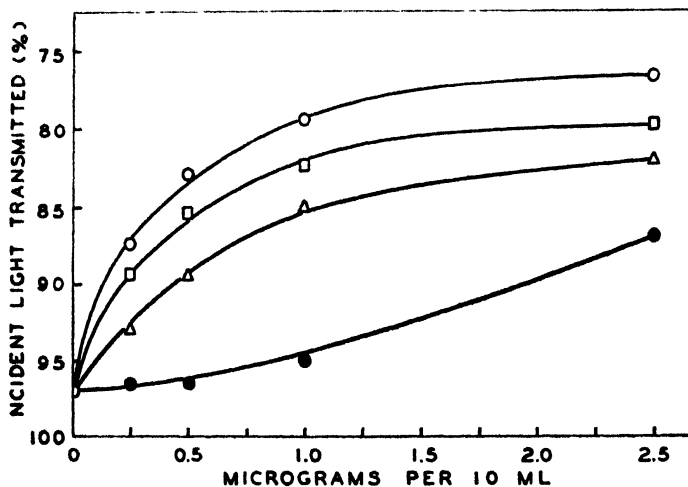


FIG. 2. The comparative response of *H. parainfluenzae* to O, putrescine; □, spermidine; Δ, spermine; and ●, agmatine.

Each of these compounds shares the 1,4-diaminobutane residue, which thus appears to be a highly specific requirement of *H. parainfluenzae* for growth. All compounds which contain this residue, however, do not possess activity; thus, arginine (present in the basal medium), ornithine, and arcaine are without activity.

At ratios of 500:1 (1  $\gamma$  of putrescine per 10 ml. of medium) none of the inactive compounds inhibited the usual growth response to putrescine.

*Comparative Activities of Active Compounds*—Quantitative response curves of *H. parainfluenzae* to increasing amounts of putrescine, spermidine, spermine, and agmatine are shown in Fig. 2. On the weight basis, putrescine is most active; however, on the molar basis putrescine, spermidine, and spermine have essentially equal activity. Agmatine is much less active. At higher levels than those shown in Fig. 2, each of the latter three compounds will permit maximum growth as follows: spermidine 5 to 10  $\gamma$ , spermine 10  $\gamma$ , and agmatine 25 to 50  $\gamma$  per 10 ml. of medium.

#### DISCUSSION

The wide distribution of spermidine and spermine in natural materials has been noted several times (10–13), but it has not been known whether the presence of these bases was incidental to, or necessary for, growth and metabolism. The importance of the results described above lies in the demonstration that these bases, or putrescine, play an essential rôle in growth of *H. parainfluenzae*, and hence, if the fundamental similarity in metabolic systems of diverse organisms is accepted, of many other organisms as well. The amounts required for growth are small, intermediate between the amounts of vitamins and of amino acids required for growth of many other microorganisms, and cannot be serving as energy sources for the organism. Hydrolysates of cells of *H. parainfluenzae* promote growth of this organism in the absence of added putrescine; preliminary results indicate that absorption of this substance from the medium occurs with considerable efficiency when it is present in concentrations which limit growth. In all of these respects, putrescine behaves like other essential nutrients. Whether the metabolically essential form for the organism is putrescine, spermidine, spermine, or some unknown derivative cannot be stated at present. All three compounds occur naturally, and although putrescine is the predominant form present in orange juice, spermine or spermidine might be a quantitatively more important form of the growth factor in some natural materials.

In many organisms, putrescine arises from ornithine by decarboxylation, and since ornithine is inactive in replacing putrescine for *H. parainfluenzae*, it may be assumed that the enzyme which catalyzes this reaction, ornithine decarboxylase, is absent. This suggests an essential rôle for this enzyme in the metabolism of other organisms, and emphasizes that the rôle of certain of the decarboxylases in microorganisms is more fundamental than has been previously suggested (14, 15).

Spermidine and spermine partially antagonize the bacteriostatic effect of atabrine and quinine on *Escherichia coli* (16, 17), and of propamidine

on certain lactic acid bacteria (18). These effects were not specific, however, since polyamines such as tetraethylenepentamine and triethylene-tetramine were also active. The latter products, as noted above, were inactive in promoting growth of *H. parainfluenzae*, whereas putrescine, which is highly active in promoting growth, showed only slight or no activity in reversing bacteriostasis. Whether the action of these compounds in reversing bacteriostasis by the antimalarial drugs is related to their nutritional rôle is not known.

#### SUMMARY

1. *Hemophilus parainfluenzae* 7901 failed to grow in a synthetic medium complete with respect to known vitamins and amino acids, but grew when this medium was supplemented with any of a variety of natural materials.

2. Properties of the unidentified factor showed it to be a stable, strongly basic amine. A highly active compound, putrescine, was isolated from orange juice in amounts which accounted for 50 per cent of the growth-promoting activity of the original juice.

3. Spermidine and spermine, which are widely distributed in nature, also show high activity for *H. parainfluenzae*; agmatine was much less active. A variety of other structurally related compounds were inactive in promoting growth. The new requirement is thus specific for certain compounds which contain the 1,4-diaminobutane residue.

4. The significance of these results is discussed briefly.

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## THE VERATRINE ALKALOIDS

### XXX. A FURTHER STUDY OF THE STRUCTURE OF VERATRAMINE AND JERVINE

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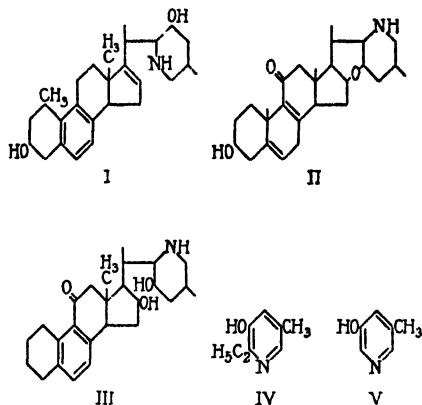
As has been reported previously (1), the most satisfactory interpretation of the major structural features of jervine,  $C_{27}H_{39}O_2N$ , is that it is a 3( $\beta$ )-hydroxy-11-keto- $\Delta^{5,8(9)}$ -steroidal secondary base in which the nitrogen atom is cyclized with carbon atoms of the isooctyl side chain. The 3rd oxygen atom appears to be of oxidic character and one point of its attachment must also be to a carbon atom of the side chain. This last conclusion was based on the isolation, from the dehydrogenation products, of a phenolic base,  $C_8H_{11}ON$ , which was suspected to be 3-methyl-6-ethyl-5-hydroxypyridine (2-ethyl-5-methyl-3-hydroxypyridine) (2) (Formula IV). The correctness of this interpretation has since been strengthened by more recent work.

Accompanying jervine in *Veratrum viride* is the secondary base veratramine,  $C_{27}H_{39}O_2N$  (3, 4). The latter contains two easily acylatable hydroxyl groups, and a double bond which can be readily hydrogenated to yield a dihydroveratramine. Studies of absorption spectra of the alkaloid and its dihydro derivative have shown that one of its rings should be benzenoid and that one of the angular methyl groups has therefore shifted in the formation of the alkaloid. Both veratramine and jervine also occur conjugated as glycosides which have been isolated in the form of the glucosides veratrosine and pseudojervine (5). In the case of the latter, only the 3( $\beta$ )-hydroxyl group, demonstrated to be present in jervine, can be involved in the conjugation and presumably in the  $\beta$  configuration. As previously reported, the attempt to show the possible presence of a 3-hydroxyl group in veratramine with aluminum *tert*-butoxide was unsuccessful. However, the presence of such a hydroxyl is to be inferred as the probable group involved in conjugation with glucose in veratrosine. Although a comparison of the rotations of the bases and their glucosides does not lend itself to the successful application of molecular rotation differences, as used by Barton (6) for simpler structural changes, nevertheless there is suggestive resemblance in the direct comparison of the rotations. Thus, on passing from jervine of  $[\alpha]_D = -147^\circ$  in ethanol to pseudojervine in ethanol-



chloroform (1:3),  $[\alpha]_D = -133^\circ$ . On passing from veratramine of  $[\alpha]_D = -69^\circ$  in methanol to veratrosine in ethanol-chloroform (1:1),  $[\alpha]_D = -53^\circ$ .

A preliminary study of the dehydrogenation of veratramine with selenium has shown that the second hydroxyl group of veratramine, as in the case of jervine, is also on the basic side chain and the evidence is very strong that it is on carbon atom 23. The dehydrogenation proceeded quite smoothly, but the fraction of most volatile simple bases appeared to be much less than has been the experience with other veratrine bases. The yield of this fraction was too small from the amount of alkaloid used to permit an attempt to isolate any methylethylpyridine, if formed. However, from the less volatile sublimate above the reaction melt an appreciable amount of a phenolic basic fraction was obtained, from which in turn a crystalline phenolic pyridine base was isolated. Analysis of this substance showed it to possess the formulation  $C_8H_7ON$ . The colors given with fer-



ric chloride and diazotized sulfanilic acid were almost identical with those obtained with the phenolic base  $C_8H_{11}ON$  from jervine. The simplest interpretation of this substance is that in its formation only the terminal methyl (carbon 27) of the side chain is retained and that it is a hydroxy- $\beta$ -picoline. There was no evidence of the formation of the  $C_8H_{11}ON$  base obtained from jervine, which must be a 3-methyl-6-ethylhydroxypyridine. From the nature of these substances as well as from the properties of the parent alkaloids, the problem of the position of the hydroxyl is left to a decision between a  $\beta$ - or  $\gamma$ -hydroxypyridine structure involving carbon atom 23 or 24.

The results of a study of the ultraviolet absorption spectra of the substances from veratramine and jervine, which were taken in methanol and in  $H_2O$  at pH about 1.8 and 11.2, are shown in the curves given in Figs. 1, 2, and 3. The shifting of the peaks with the change of pH shows a sufficient agreement with previous experience (7-9) to indicate that they are  $\beta$ -

hydroxypyridines and not  $\alpha$ - or  $\gamma$ -hydroxypyridines. To satisfy this requirement, barring most unlikely rearrangements during the dehydrogenation process, the hydroxyl group is therefore on carbon atom 23 of the steroid side chain. A substantiation of the  $\beta$ -hydroxypyridine character was obtained in the color reactions produced with the Folin-Denis reagent. As shown by Kuhn and Wendt (7), a positive reaction is given only by  $\beta$ -

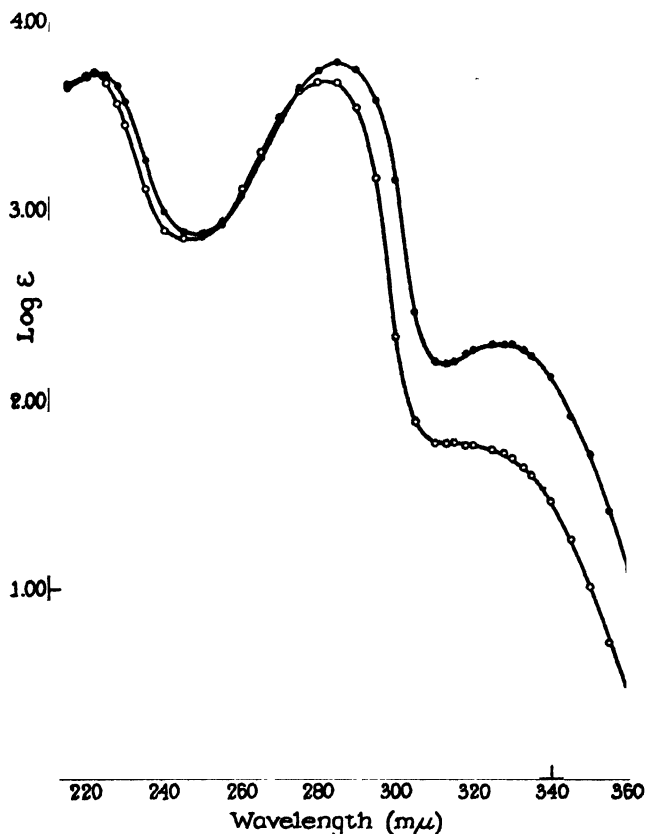


FIG. 1. O =  $C_6H_7ON$  from veratramine; ● =  $C_8H_{11}ON$  from jervine; in methanol.

hydroxypyridines and not by  $\alpha$  or  $\gamma$  derivatives. The blue color obtained with the  $C_6H_7ON$  base from veratramine was of a lighter shade than that from the jervine product,  $C_8H_{11}ON$ , and this in turn was less intense than the deep color given by pyridoxine, which was used for comparison. An attempt is in progress to synthesize these bases for substantiation of Formulas IV and V.

The investigation of the residue which remained undistilled in the de-

hydrogenation of veratramine is still in a preliminary stage. However, the neutral hydrocarbon fraction, which was separated through alumina, has readily yielded in the first fractions a crystalline hydrocarbon (m.p., 153.5–155.5°). This was indistinguishable in properties from the hydrocarbon  $C_{22}H_{20}$  (m.p., 154–155°) previously obtained from jervine (2). Like the jervine hydrocarbon, it failed to yield a stable picrate and gives a strong

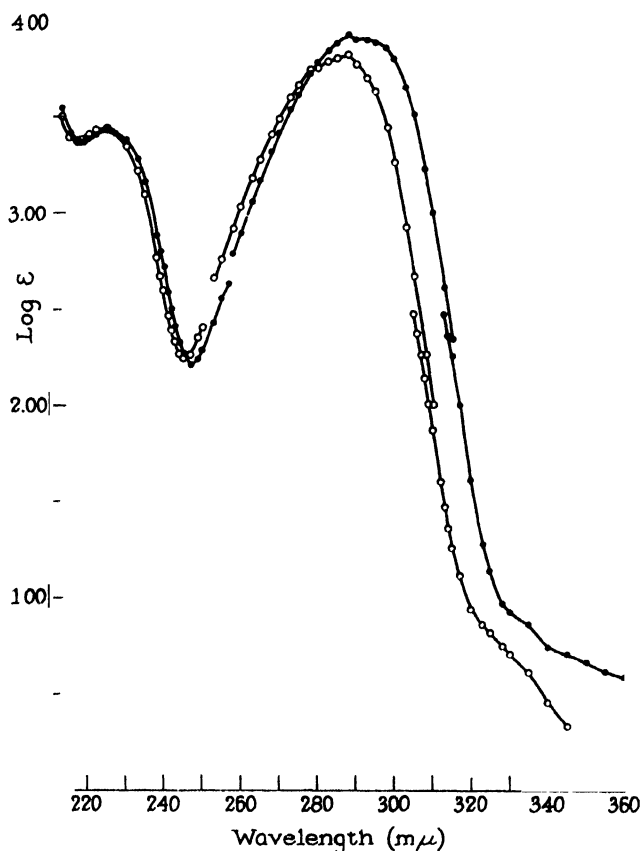


FIG. 2. O =  $C_6H_7ON$ ; ● =  $C_6H_{11}ON$ ; both in aqueous HCl of pH 1.85

test with tetranitromethane. The absorption spectrum shown in Fig. 4 is also in essential agreement with that previously found with the jervine product. In the earlier work a resemblance to the absorption curve of 1,2-benzofluorene was discussed. However, the curve obtained is not incompatible with that to be expected of a chrysene homologue and this would be more consistent with recent data regarding the structure of jervine. It is now planned to complete the study of these hydrocarbons.

From the data obtained a possible interpretation of the structure of veratramine is presented in Formula I. The close similarity of the ultra-violet absorption curves of veratramine and dihydroveratramine (4) already published shows that the reactive double bond is separate and not conjugated with those of the benzenoid ring. A recent determination in chloroform solution has shown for veratramine a rotation of  $[\alpha]_D^{27} = -71.8^\circ$  ( $c =$

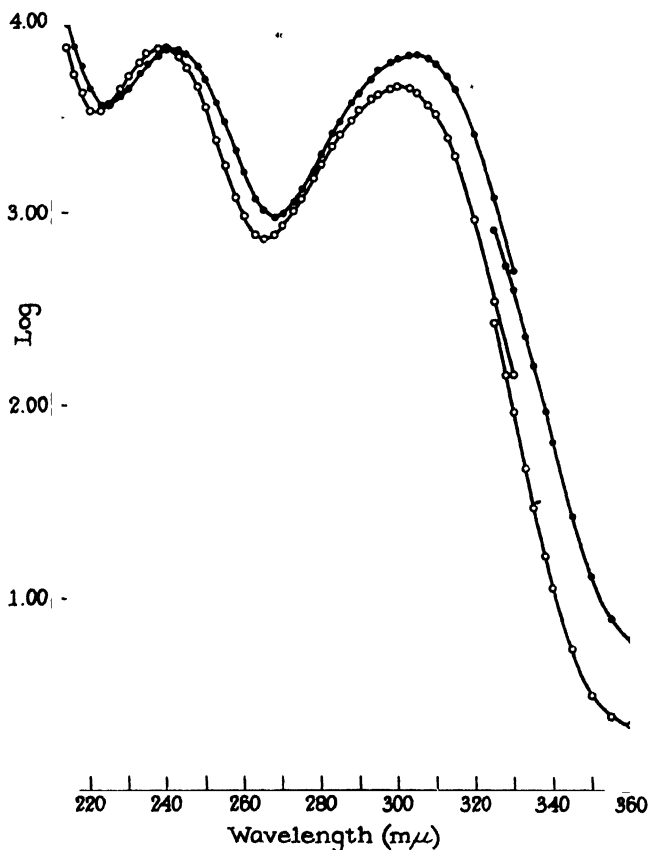


FIG. 3.  $\circ = C_6H_7ON$ ;  $\bullet = C_6H_{11}ON$ ; both in aqueous NaOH of pH 11.2

0.99) and for dihydroveratramine,  $[\alpha]_D^{27} = +27.4^\circ$  ( $c = 0.95$ ). The molecular rotation difference from this of  $-407^\circ$  to be assigned to the double bond has not been interpreted from the available data. We are dealing with a benzenoid derivative. The  $\Delta$  of  $-298^\circ$  shown by Barton and Klyne (10) for the  $\Delta^5$  bond is the nearest approach to the above difference. If the usual 3( $\beta$ )-hydroxy- $\Delta^5$ -stenol character is to be assigned to veratramine, it would require that Ring C is benzenoid. However, it appears doubtful

that a  $\Delta^5$  double bond could resist a shift to  $\Delta^6$  for conjugation with such a near-by benzenoid Ring C. If Ring B is assumed to be benzenoid, it is similarly true that a double bond at  $\Delta^{16}$  should readily shift to  $\Delta^{14}$  to conjugate with Ring B. However, with  $\Delta^{16}$  or  $\Delta^{17}$  <sup>(20)</sup> there might be less likelihood of double bond shift. But as a possible objection the molecular rotation contributions of such double bonds as listed by Barton and Klyne (10) and Fieser and Fieser (11) in otherwise saturated substances are  $+31^\circ$

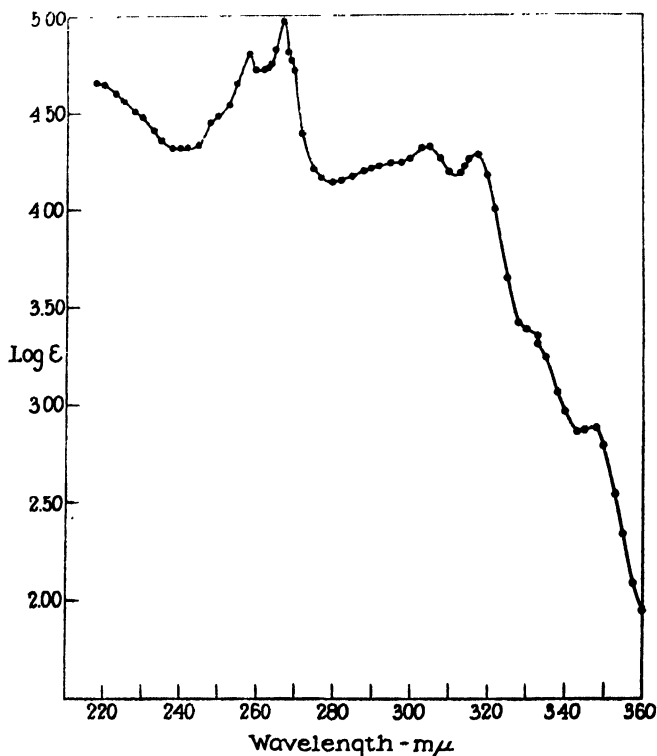


FIG. 4 ● =  $C_{22}H_{20}$  hydrocarbon

for  $\Delta^{16}$  and  $+35^\circ$  for  $\Delta^{17}$  <sup>(20)</sup>. If not in the side chain, a further possibility is a  $\Delta^5$  base with Ring D enlarged and benzenoid.

In the case of jervine, with one place of attachment of the oxidic linkage probably established on  $C^{23}$ , there remains in question its other point of attachment. In the formation of isojervine it appears from its resistance to hydrogenation that a benzenoid structure has developed, even though the presence of the latter is not apparent in its ultraviolet absorption spectrum and is possibly masked by other contributing factors. For

further information a preliminary study was made of its behavior on selenium dehydrogenation. However, the instability of isojervine in a different sense resulted in a large amount of apparent resinification. From the volatile basic material a small phenolic basic fraction was separated, which in turn yielded only a very small amount of the methylethyl-hydroxypyridine originally obtained from jervine. There was no evidence of the formation of the hydroxypicoline characteristic of veratramine. In view of the negative outcome of other studies of isojervine, its correct interpretation has not been completed. There remains the possibility that it retains the carbonyl group and that the isomerization involves removal of the 3-hydroxyl group, with double bond formation, and production of a benzenoid structure of Ring B, with accompanying rearrangement of the angular methyl group. Simultaneously, the two acylatable hydroxyl groups of isojervine may arise from cleavage of the oxidic bridge which may be more labile in jervine than in its hydrogenation products. Such structures would be represented by Formulas II and III for jervine and isojervine respectively.

This possibility is supported by the previously reported experience with the hydrolysis of pseudojervine (5). With 2 per cent HCl, cleavage occurred, with the production of a large fraction of isojervine and no jervine. Under similar conditions jervine itself was converted to isojervine to the extent of less than one-third, and the major part was recovered unchanged. This might suggest that during hydrolysis of the glycoside the liberated 3-hydroxyl group is activated and participates more readily in the double bond formation. There is some analogy for the loss of this hydroxyl in the conversion of periplogenin into trianhydroperiplogenin (12).

In the case of jervine the possibility of an oxygen bridge between carbon atoms 23 and 21 or between carbons 23 and 27 must be considered. But the production of 3-methyl-6-ethylpyridine and its 5-hydroxy derivative on dehydrogenation and the failure to detect any bases with oxygen attached to the side chains, as in the case of cevine (13), would appear to be against such an arrangement. An oxygen bridge to carbon 26 appears to be definitely excluded by the character of isojervine and its stability to acids.

#### EXPERIMENTAL

*Dehydrogenation of Veratramine*—A ground mixture of 5 gm. of veratramine and 15 gm. of selenium contained in a flask, through which a very slow stream of nitrogen passed, was placed in a bath at 240°. The temperature during 20 minutes was gradually raised to 280° to permit water to distil off. During the following 30 minutes the temperature reached 300°. From the molten mass there was considerable gas evolution. Aside from the small amount, apparently water, very little condensed at first. After

20 minutes the temperature was gradually raised to 330–335° and maintained at this point for 2½ hours. Gas evolution had subsided and a small amount of colored material had collected in the chilled receiver, although a less volatile and little colored product had accumulated in the upper portion of the flask. The distillate was worked up separately from the condensation product. After removal of some colored distillate with ether from the side arm of the flask, the washings were added to the few drops of distillate in the receiver. This extract (Fraction A) will be discussed below. The less volatile condensate (Fraction B) in the reaction flask above the solidified melt was carefully dissolved with ether, contact with the lower solid being avoided. The yellow ether solution was filtered from selenium and shaken with 2 cc. of 10 per cent HCl. The aqueous phase became a deep red, with precipitation of colored resinous salts. This was followed by a second 2 cc. of HCl and then repeatedly with small portions of water, which finally redissolved practically all of the solid. The remaining ether solution, which possibly contained some hydrocarbons and other neutral material, was not studied further.

The HCl solution was treated with excess 25 per cent NaOH until strongly alkaline, with accompanying color change to yellow. The turbid mixture, which smelled of pyridine homologues, was repeatedly extracted with ether and this extract was joined with a similar fraction to be described later in connection with Fraction A. The alkaline aqueous phase was saturated with CO<sub>2</sub> and then repeatedly extracted with ether. The dried extract yielded 0.125 gm. of a slightly colored oil, which crystallized from a small volume of ether as compact aggregates of wedge-shaped micro platelets. The yield of this fraction was 40 mg., and an additional 10 mg. were obtained from the mother liquor. After recrystallization from benzene it melted at 137.5–139° (corrected). When mixed with the C<sub>8</sub>H<sub>11</sub>ON base from jervine previously described (2) (m.p. 145–147°), a marked depression was obtained. It was easily soluble in water, methanol, and ethanol.

C <sub>8</sub> H <sub>7</sub> ON.	Calculated.	C 66.02,	H 6.47
	Found. (a)	“ 66.40,	“ 6.72
	(b)	“ 65.96,	“ 6.53

The aqueous solution gave a brown-orange color with ferric chloride and coupled in alkaline solution with diazotized sulfanilic acid to a brown-yellow solution. This changed to a bright yellow on acidification, but returned to the former color with alkali. No difference was noted in parallel reactions with the substance from jervine.

The mother liquors of this hydroxypicoline contained much oily material, which was not studied further.

The above ether solution of Fraction A obtained from the reaction distil-

late was similarly treated to separate the basic phenolic fraction. The latter amounted to 60 mg. of resinous material. From the concentrated ether solution 5 mg. of crystalline phenolic base, identical with the above hydroxypicoline, were obtained.

The ether solutions of non-phenolic bases from Fractions A and B were joined and on concentration yielded a thick, dark brown oil with an odor suggestive of the higher pyridines. When the material was treated again with ether, some undissolved substance remained, and the filtrate on concentration gave about 50 mg. of residue. When dissolved in alcohol and treated with 50 mg. of picric acid in alcohol, a dark tar precipitated from which no crystalline material could be obtained. The supernatant solution yielded on concentration a small amount of tar mixed with crystals, which were collected with alcohol. On recrystallization from acetone, 4 mg. of light yellow needles were obtained which melted at 224–227°. This substance has not been identified.

Found, C 44.09, H 3.60

The attempt to obtain methylethylpyridine from this fraction was unsuccessful.

The undistilled residue which remained in the original dehydrogenation flask was extracted with benzene. The filtered dark brown solution was extracted with 5 cc. of 10 per cent NaOH. An appreciable amount appeared to dissolve, with some slight tar formation. This was followed by repeated water extraction. The alkaline aqueous extract was saturated with CO<sub>2</sub>, which caused some precipitation and foaming, and then repeatedly extracted with ether. This yielded 0.15 gm. of a colored resin. The latter was not completely studied beyond the isolation of a small amount of hydroxypicoline.

The remaining benzene phase was shaken with 5 cc. of 10 per cent HCl, which caused precipitation of a dark red tar of salts. The acid extract was drained off and the mixture was again shaken with 5 cc. of 10 per cent HCl. This was followed progressively with 5 cc. portions of water until the latter became but little colored. However, most of the red tar of salts remained undissolved on the funnel walls and the benzene phase was separated, followed by rinsings. The HCl salt fraction was dissolved in methanol and the red solution was cleared from suspended material by centrifugation. The concentrated solution when made alkaline changed to a brown color. Extraction with benzene yielded 1.15 gm. of a colored resin of bases. This material in acetone yielded 0.17 gm. of a crystalline product, m.p. 248–250°, which appears to be a phenolic base and of the general order of cevanthridine. A report on this substance will be presented in a later paper.

The dried benzene solution, which contained essentially neutral material,



yielded 2.23 gm. of a dark colored tar. A solution of this in 15 cc. of benzene was chromatographed through 60 gm. of  $\text{Al}_2\text{O}_3$  and developed with benzene. The color spread down in bands and the material quickly emerged as a yellow solution. At this point the first 15 cc. yielded 0.15 gm., which did not crystallize. The following 10 cc. contained 0.6 gm. of a partly crystalline resin. The third 10 cc. gave 0.29 gm., which crystallized more copiously, and the fourth 10 cc. gave 0.11 gm. of partly crystalline material. The following 10 cc. fractions eluted progressively less material, which from the tenth to the eighteenth fractions remained almost constant at about 20 mg. and again gradually crystallized. More material was finally obtained in fractions with 1 per cent methanol in benzene. The study of these later fractions has been postponed.

The second fraction was crystallized from a small volume of benzene at  $0^\circ$  and collected in the cold with a little toluene. 72 mg. were obtained. The third and fourth fractions yielded 45 and 28 mg., respectively. Further study showed them to be essentially identical. After repeated recrystallization from benzene-alcohol and then twice from ether, the substance formed long diamond-shaped leaflets which melted at  $153.5\text{--}155.5^\circ$ . The mixture with the  $\text{C}_{22}\text{H}_{20}$  hydrocarbon from jervine (2) sintered at  $151^\circ$  and melted at  $153.5\text{--}155.5^\circ$ . The jervine product taken again for comparison sintered at  $152^\circ$  and melted at  $153.5\text{--}155.5^\circ$ . The ultraviolet absorption spectrum is shown in Fig. 4.

$\text{C}_{22}\text{H}_{20}$ .	Calculated.	C 92.91, H 7.09, mol. wt. 284.18
	Found. (a)	" 92.55, " 7.27 " " 274, 267.3
	(b)	" 92.82, " 6.89

Although concentration of a solution of the hydrocarbon and picric acid in acetone gave a red color, only leaflets of the hydrocarbon crystallized. When collected in three successive fractions, practically all of the hydrocarbon was recovered. Similarly, from benzene no picrate was obtained. With trinitrobenzene there appeared to be little tendency to form a stable compound.

*Dehydrogenation of Isojervine*—A mixture of 5 gm. of isojervine and 15 gm. of selenium was heated as in the previous case at first at  $240^\circ$ , and then the temperature was gradually raised to  $325\text{--}330^\circ$  and held at this point for 2 hours. At about  $310^\circ$  an unusual amount of almost black material filled the upper portion of the apparatus and considerable dark oil accumulated in the receiver. When the distillate was dissolved in ether, the basic fraction was extracted with HCl. From this solution the bases were liberated with an excess of NaOH and reextracted with ether. The mixture smelled distinctly of pyridine bases and was not studied further. The NaOH solution, after neutralization with  $\text{CO}_2$ , was in turn extracted with ether. From this only 15 mg. of a red-brown resin were obtained. The solution in

ether, when cleared with norit and concentrated, yielded characteristic, almost rectangular leaflets. 3 mg. were collected with ether. The substance melted at 142–143° and, when mixed with the jervine product (m.p. 145–147°), there was no appreciable depression. When mixed with the hydroxypicoline from veratramine, a marked depression was obtained.

$C_8H_{11}ON$ . Calculated. C 70.02, H 8.08; found, C 69.75, H 7.89

Investigation of the undistilled residue in the reaction flask for additional phenolic basic material gave only about 15 mg. of colored resin, from which none of the  $C_8H_{11}ON$  compound could be crystallized.

No attempt has been made to investigate the other fractions of the dehydrogenation.

All of the analytical data have been furnished by Mr. D. Rigakos of this laboratory.

#### SUMMARY

The dehydrogenation of veratramine has yielded a phenolic base,  $C_8H_7ON$ , which is closely related to the phenolic base,  $C_8H_{11}ON$ , previously obtained from jervine. The change in the ultraviolet absorption spectra of these substances with change of pH, as well as a positive color reaction, given with the Folin-Denis reagent, affords the strongest evidence that they are  $\beta$ -hydroxypyridine bases, and that the former is 3-methyl-5-hydroxypyridine and the latter 3-methyl-6-ethyl-5-hydroxypyridine. The production of these substances in conjunction with other data has permitted certain conclusions regarding the structure of the parent steroidal bases.

The hydrocarbon  $C_{22}H_{20}$ , previously obtained from jervine, has also been found with other crystalline substances among the dehydrogenation products of veratramine.

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# ANAEROBIC PHOSPHORYLATION DUE TO THE DISMUTATION OF $\alpha$ -KETOGLUTARIC ACID IN THE PRESENCE OF AMMONIA\*

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Reaction (1), the dismutation of  $\alpha$ -ketoglutaric acid in the presence of ammonia to yield succinic acid, carbon dioxide, and glutamic acid, was reported by Krebs and Cohen (1) to occur in kidney and heart but not in liver. In the present work washed insoluble particles from rat liver were



found to catalyze this dismutation. Phosphorylation has been found to be coupled with the dismutation, and this coupled reaction has been studied quantitatively. While this work was in progress, Krebs, Eggleston, and Hems (2) reported that the dismutation can be studied in liver homogenates, but that little reaction occurs in liver slices. Although the phosphorylation is readily demonstrated with washed liver particles, as reported below, it would be less practical to study the coupled phosphorylation in whole liver homogenates because of many side reactions.

## EXPERIMENTAL

*Materials and Methods*—The sources of materials were the same as for a previous investigation (3), except that in most cases the suspension of liver particles, which contained the enzyme system, was washed only twice instead of three times. The  $\alpha$ -ketoglutaric acid was measured by the method of Friedemann and Haugen (4). The glutamic acid was determined as  $\alpha$ -amino acid according to Moore and Stein (5) after aeration to remove ammonia (at about pH 12). There is no reason to suspect the formation of significant amounts of any free amino acid except glutamic acid. The ammonia was trapped in 0.05 N  $\text{H}_2\text{SO}_4$  and measured by the same colorimetric ninhydrin method. Other determinations were made as previously described (3).

The dismutation, as a rule, was followed manometrically by carbon dioxide evolution from bicarbonate buffer (1). The balance experiment was carried out in Thunberg tubes.

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### Results

*Occurrence of Dismutation*—In anaerobic experiments very little reaction occurs when  $\alpha$ -ketoglutarate is added to washed liver particles (Table I). This suggests that the enzymes essential for dismutation of  $\alpha$ -ketoglutarate to succinate, carbon dioxide, and  $\alpha$ -hydroxyglutarate are not all present. However, when ammonium ion is added along with the  $\alpha$ -ketoglutarate, carbon dioxide liberation and removal of inorganic phosphate take place

TABLE I

#### *Phosphorylation Due to Dismutation of $\alpha$ -Ketoglutarate*

The final reaction mixture contained 0.033 M  $\text{KHCO}_3$ , 0.012 M potassium phosphate buffer, pH 7.6, 0.006 M  $\text{MgCl}_2$ , 0.04 M NaF, 0.04 M glucose, 0.0015 M adenosine triphosphate, 0.0002 M diphosphopyridine nucleotide, 0.0001 M cocarboxylase, yeast hexokinase, and rat liver enzyme. Final volume 3.0 ml.; gas phase 95 per cent  $\text{N}_2$  and 5 per cent  $\text{CO}_2$ ; incubated 30 to 45 minutes at 30°. All the values are in micromoles. The carbon dioxide evolved has been corrected for retention by buffers in the medium.

Experiment No.	$\alpha$ -Ketoglutarate added	Ammonium ion added	$\text{CO}_2$ evolved	$\text{CO}_2$ , $\Delta$	Inorganic phosphate removed	P: $\text{CO}_2$ ratio
1			2.8			
		50	2.9			
	30		4.2			
	30	2	7.1	2.9	0.8	
	30	10	15.0	10.8	7.4	0.69
	30	50	19.4	15.2	9.3	0.61
2	30	50	17.9	13.7	9.9	0.72
	40		2.2			
	40	2	4.1	1.9		
	40	4	6.5	4.3		
	40	8	9.9	7.7		
	40	12	14.1	11.9		
3	20	30	9.4	7.2		
	50	80		8.4	5.5	0.66
4	50	80		10.0	9.8	0.98
5	50	80		6.3	5.5	0.86
6	50	80		19.9	13.9	0.70
Average						0.75

at a rapid rate (Table I). Experiments 1 and 2 indicate that the ammonia enters into the reaction stoichiometrically rather than catalytically, for the reaction stops when 1 molecule of carbon dioxide has been evolved per molecule of ammonia originally present. This is in quantitative agreement with the postulated reaction. Similarly, if ammonia is in excess and the  $\alpha$ -ketoglutarate becomes limiting, the reaction stops when a little less than 1 molecule of carbon dioxide has been evolved for each 2 molecules of

$\alpha$ -ketoglutarate added. This value corresponds to that expected from almost complete utilization of  $\alpha$ -ketoglutarate in the dismutation.

*Phosphorylation Coupled with Dismutation*—Purified yeast hexokinase<sup>1</sup> and glucose were added in all experiments. The removal of inorganic phosphate which is linked with the dismutation is also shown in Table I. This phosphate is converted to an organic form corresponding to glucose-6-phosphate in its resistance to acid hydrolysis. Acid hydrolysis was carried out on nearly every experiment as a check on the possible formation of inorganic pyrophosphate. It will be noted that an average of 0.75 molecule of phosphate is esterified per molecule of carbon dioxide evolved.

TABLE II  
*Balance Experiment*

The reaction mixture was similar to that in Table I, with the omission of  $\text{KHCO}_3$ . The Thunberg tubes were evacuated and flushed with nitrogen several times. 55 minutes at 20°. All the values are in micromoles.

	$\alpha$ -Ketoglutarate		Succinate		Ammonia		$\alpha$ -Amino acid (glutamate)		Inorganic phosphate	
		$\Delta$		$\Delta$		$\Delta$		$\Delta$		$\Delta$
$\alpha$ -Ketoglutarate + $(\text{NH}_4)_2\text{SO}_4$ , not incubated	45.1		0		74.3		6.3		61	
No substrate, incubated	0.6		0.2		10.6		8.6		69.9	
	0.3		0		9.0		8.1		70.5	
$(\text{NH}_4)_2\text{SO}_4$ , incubated	0.2		0		78.5		10		69.8	-0.4
	0.2		0		83.8		9.8		72.6	+2.4
$\alpha$ -Ketoglutarate, incubated	35.5	-9.6	3.7	+3.7	3.0	-7.6	11.1	+2.7	65	-5.2
	39.2	-5.9	2.6	+2.6	3.4	-5.6	11.7	+3.3	66.1	-4.1
$\alpha$ -Ketoglutarate + $(\text{NH}_4)_2\text{SO}_4$ , incubated	5.6	-39.5	20.2	+20.2	56.8	-23.8	26.8	+16.9	52.7	-17.5
	8.8	-36.3	24.2	+24.2	58.2	-22.4	27.6	+17.7	53.2	-17.0

If one makes allowance for some loss in side reactions, the true value would appear to be 1.0.

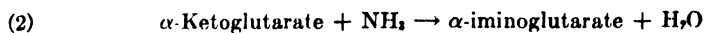
*Chemical Balance*—A balance experiment was carried out to demonstrate the actual reaction occurring and therefore responsible for the observed phosphorylation. The data are presented in Table II. The removal of 2 molecules of  $\alpha$ -ketoglutarate and 1 molecule of  $\text{NH}_3$ , with the formation of 1 molecule of succinate and 1 of glutamate, and the occurrence of one phosphorylation are rather clearly indicated. The agreement between values is very good, except that the succinate values are a little high. The increase in inorganic phosphate upon incubation of the mixture with-

<sup>1</sup> The authors are indebted to Anheuser-Busch, Inc., for considerable amounts of bakers' yeast used as a source for hexokinase.

out substrate ( $9\ \mu\text{M}$ ) in this particular experiment was twice as great as that found in the average experiment. The relatively small amount of reaction which occurs when  $\alpha$ -ketoglutarate alone is added may well be due to ammonia in the tissue or released during the incubation. By actual determination  $9.8\ \mu\text{M}$  of ammonia were present in the complete controls at the end of the incubation, while only  $3.2\ \mu\text{M}$  were left in the tubes when  $\alpha$ -ketoglutarate (but no extra ammonia) was included.

#### DISCUSSION

The actual mechanism probably involves iminoglutarate as an intermediate, as suggested by Krebs and Cohen (1).



The point of origin of the high energy phosphate bond which is transferred over the adenylate system to glucose is not determined by these experiments, but the most likely possibility is in the  $\alpha$ -ketoglutarate to succinate reaction, since it seems unlikely that sufficient energy release would occur in reaction (4), and reaction (2) is probably non-enzymatic. The identity of any intermediate electron-transferring coenzyme remains to be established. While the present experiments demonstrate that the dismutation is catalyzed by the insoluble particles from liver and that phosphorylation can be linked with the reaction, the actual quantitative rôle of such reactions in the metabolism of aerobic cells may not be large. However, the reaction and the resulting phosphorylation are of the greatest immediate concern in trying to determine the number of phosphorylations which are coupled with the oxidation of  $\alpha$ -ketoglutarate to succinate aerobically. The occurrence of any of the possible dismutations during aerobic experiments, by contributing to the removal of inorganic phosphate but not to the oxygen consumption, would tend to give falsely high P:O ratios (inorganic phosphate removed to atoms of oxygen consumed).

Since three washings of the tissue particles frequently yielded inactive preparations, while two washings always resulted in active preparations, it seems possible that part of the system necessary for conversion of  $\alpha$ -ketoglutarate to glutamate may be removed. It is known from other work that three washings leave the  $\alpha$ -ketoglutarate oxidizing system intact.

#### SUMMARY

1. The dismutation of  $\alpha$ -ketoglutarate in the presence of ammonia to yield succinate, carbon dioxide, and glutamate is catalyzed by washed insoluble particles from homogenized rat liver.

2. Phosphorylation of glucose via the adenylate-hexokinase system can be coupled with the dismutation. Probably one phosphorylation occurs per molecule of  $\alpha$ -ketoglutarate oxidized to succinate.

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## PHOSPHORYLATION COUPLED WITH THE OXIDATION OF $\alpha$ -KETOGLUTARIC ACID\*

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Present evidence indicates that the energy released by cellular oxidations is in part captured in the form of high energy phosphate bonds, and that such phosphorylations represent the major, if not the only, way in which oxidative energy is made available for the performance of the various functions of the cell. Thus it is of the greatest interest to determine the number of high energy phosphate bonds which can be created by the oxidation of each of the various foodstuffs and metabolites.

The question of the number of energy-rich phosphate bonds which could be generated by the single step oxidation of  $\alpha$ -ketoglutarate to succinate and the passage of the electrons over the electron transport systems to oxygen was investigated several years ago by Ochoa (1), who concluded that the number was probably 3.0. This figure was consistent with the value of 3.0 earlier estimated to be the ratio of phosphorylations to atoms of oxygen consumed in the complete oxidation of pyruvate to carbon dioxide and water. These values have been widely accepted and used in profitable speculation about the phosphorylations coupled with oxidations in the tricarboxylic acid cycle. Although values as high as those of Ochoa have been seriously questioned by Ogston and Smithies (2), more recently Loomis and Lipmann (3) and Cross *et al.* (4) have published data which suggest that Ochoa's figure is in the correct range. The present paper presents further data obtained with the washed liver particles used in earlier studies. The final experimental conditions were such as to reduce greatly the side reactions which would otherwise result in loss of a considerable fraction of esterified phosphate. In Ochoa's investigation the directly observed P:O ratios averaged less than 2.0 with heart muscle suspensions, whereas conditions have now been found under which it is possible to get directly determined P:O ratios of 3.4 to 3.5 with washed liver particles. This suggests that the true value in cellular metabolism may be 4.0.

\* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

## EXPERIMENTAL

*Materials and Methods*—The sources of materials<sup>1</sup> and the methods were those previously used (5), except that the  $\alpha$ -ketoglutaric acid was determined by only one method, that of Friedemann and Haugen (6).

*Experimental Conditions*—Experiments were carried out in a standard Warburg apparatus with the bath temperature set at 15°. In order to avoid formation of precipitates of magnesium-fluoride-phosphate compounds, only a small amount of magnesium was included in the buffer medium. The major portion of the magnesium indicated by the final concentrations was mixed with the tissue particle suspension. This mixture constituted the last addition to the Warburg vessel. The substrate was tipped in after equilibration at 15°.

*Results*

*Temperature*—When an incubation temperature of 30° was used in studying phosphorylation due to  $\alpha$ -ketoglutarate oxidation, P:O ratios varied widely from 2.0 to 2.8 to an occasional 3.5. At first it was believed that the values over 3.0 were due to unrecognized errors. However, as the study continued, it appeared that the phosphorylative mechanisms usually were inactivated more rapidly than the oxidative mechanisms. More credence was therefore attached to the possible validity of the high P:O values. Several experimental procedures were tried in an effort to find conditions which would produce maximal phosphorylating activity. The simple procedure of lowering the temperature to 15° for incubation resulted in experiments which consistently, without a single exception, showed uncorrected P:O ratios greater than 3.0 (average 3.7). There seems little doubt that lowering the temperature is of great importance for preventing loss of part of the phosphorylating mechanisms. Theoretically the lower temperature might also alter the proportion of phosphate bonds split in side reactions. However, there is reason to believe that losses in side reactions are minimal. Like Cross *et al.* (4) and Lehninger (7), we found that incubation at 30° without substrate results in some loss of phosphorylating activity. It is clear that phosphorylation and oxidation are easily dissociated, since with prolonged storage the ability to phosphorylate may be completely lost, even though the capacity to oxidize substrate remains. It is thus important to prevent such dissociation if maximal P:O ratios are to be obtained.

*Possible Sources of Error Which Were Controlled.* (1) *Inorganic Pyrophosphate Formation*—Routine acid hydrolysis was carried out as a check on possible formation of inorganic pyrophosphate, since formation of this

<sup>1</sup> The authors are indebted to Anheuser-Busch, Inc., for considerable amounts of bakers' yeast used as a source for hexokinase.

substance might yield falsely high P:O ratios, due to the removal of 2 inorganic phosphate molecules, while only 1 energy-rich phosphate bond was being formed. Since the increase in inorganic phosphate on 10 minutes hydrolysis in 1 N  $\text{H}_2\text{SO}_4$  was in general of the same order of magnitude in the controls and in the samples with substrate, and since the increase approximated that expected from the adenosine diphosphate which might be present, it was concluded that inorganic pyrophosphate was not formed under the conditions used. This same conclusion has been reached by Cross *et al.* (4), who found no pyrophosphate when hexokinase and glucose were used.

(2) *Phosphorylation Due to Dismutation of  $\alpha$ -Ketoglutarate*—This type of reaction, exemplified by the one discussed in the preceding paper (8), would cause falsely high P:O values if it occurred simultaneously with the oxidation, for it would contribute to inorganic phosphate removal but not to the oxygen consumption. Anaerobic controls were run simultaneously with the aerobic experiments (Table I). These indicated a small amount of inorganic phosphate removal in some experiments, with almost negligible amounts in others.

(3) *Phosphorylation Due to Dismutation of  $\alpha$ -Ketoglutarate Followed by Oxidation of Glutamate*—This sequence of reactions would result in one phosphorylation due to the dismutation, and possibly three due to glutamate oxidation, yielding a P:O ratio of 4.0. Glutamate is readily oxidized by the enzyme preparation. A small amount of ammonia might act catalytically to permit the dismutation, for ammonia would be regenerated in the oxidation of the glutamate. This possibility would not be ruled out by the anaerobic controls mentioned above, for in them the ammonia would not be regenerated. When the possible occurrence of these reactions was checked by adding 30  $\mu\text{M}$  of ammonia in both the aerobic and the anaerobic parts of the experiment, the removal of  $\alpha$ -ketoglutarate and inorganic phosphate in each case was the same as if no ammonia had been added (Table I). These findings indicate that very little dismutation occurred even when ammonia was added in large amounts. Such a conclusion is consistent with the observation made previously (8) that liver particles washed three times usually would not catalyze the dismutation.

(4) *Glycolytic Phosphorylation*—There is no evidence for phosphorylation from glycolysis of glucose or traces of glycogen which might be present in the tissue, as indicated by the controls without  $\alpha$ -ketoglutarate as substrate. These all contained glucose, hexokinase, and amounts of adenosine triphosphate sufficient for priming the reaction; yet inorganic phosphate increased, instead of decreasing, during incubation due to phosphatase action. However, the possibility still existed that some of

the much larger amounts of glucose-6-PO<sub>4</sub> formed in the vessels with  $\alpha$ -ketoglutarate might be converted to hexose diphosphate and undergo

TABLE I

*Phosphorylation Due to Oxidation of  $\alpha$ -Ketoglutarate*

The final reaction mixture contained approximately 0.025 M potassium phosphate buffer, pH 7.5, 0.04 M NaF, 0.006 M MgCl<sub>2</sub>, 0.04 M glucose, 0.0015 M adenosine triphosphate, 0.0002 M diphosphopyridine nucleotide, 0.0001 M cocarboxylase, cytochrome c, yeast hexokinase, and rat liver enzyme. Final volume 2.7 ml. 30 to 35  $\mu$ M of  $\alpha$ -ketoglutarate as indicated. Incubation aerobically or anaerobically as indicated for 50 to 75 minutes at 15°. All the values are expressed as micromoles. "Phosphate" always refers to inorganic phosphate. Each experiment was performed in duplicate throughout.

	Experiment No.					
	1	2	3	4	5	6*
Phosphate before incubation	59.6	65.2	62.2	63.1	63.9	65.5
Aerobic oxidation						
Oxygen consumed	4.6	5.6	5.5	5.8	5.2	5.6
Phosphate after incubation without substrate	61.3	68.6	66.3	67.7	69.1	71.6
Phosphate after incubation with substrate	25.5	27.0	23.7	26.6	30.9	30.1
$\Delta$ phosphate due to $\alpha$ -ketoglutarate	-35.8	-41.6	-42.6	-41.1	-38.2	-41.5
P:O ratio	3.9	3.7	3.9	3.5	3.7	3.7
$\alpha$ -Ketoglutarate removed		12.9	12.3	11.9	10.1	13.5
Succinate formed				7.5	7.9	
Anaerobic dismutation						
Phosphate after incubation without substrate	59.2	67.9	64.7	66.1	68.8	68.9
Phosphate after incubation with substrate	56.2	62.9	62.9	64.8	66.8	67.1
$\Delta$ phosphate due to $\alpha$ -ketoglutarate	-3.0	-5.0	-1.8	-1.3	-2.0	-1.8
$\alpha$ -Ketoglutarate removed†		1.7	2.7	3.3	0.8	4.4
Glycolytic phosphorylation, 30 $\mu$ M fructose-1,6-diphosphate						
Phosphate before incubation				74.8	72.6	
Phosphate after incubation				77.3	76.8	
P:O ratio corrected for anaerobic removal of phosphate‡	3.6	3.3	3.7	3.4	3.5	3.5

\* 30  $\mu$ M of NH<sub>4</sub>Cl added in this experiment.

† Amount too small to be determined with accuracy.

‡ In Experiments 1, 2, and 3 the P:O ratios were 3.1, 3.2, and 3.2 with malonate present.

glycolysis. When this was checked by adding hexose diphosphate, no glycolytic phosphorylation was observed (Experiments 4 and 5, Table I).

**Balance Experiments**—The removal of  $\alpha$ -ketoglutarate agreed fairly well with the atoms of oxygen consumed, but was usually a little larger (Table I). About 75 per cent of the  $\alpha$ -ketoglutarate disappearing could be recovered as succinate, as shown in Experiments 4 and 5.

**P:O Ratios**—The directly determined ratios for moles of inorganic phosphate to atoms of oxygen consumed (Table I) were 3.5 to 3.9 (average 3.7). With corrections for anaerobic phosphorylation the average is reduced to 3.4 to 3.5. As noted in the "Discussion," this anaerobic correction may be too large. In order to err, if at all, on the conservative side, no allowance is made for possible splitting of phosphate bonds in side reactions. The presence of the hexokinase system, of course, greatly reduces the losses through such side reactions, since adenosine triphosphate is not allowed to accumulate.

#### DISCUSSION

Ideally one should study the yield of phosphorylation from a single one-step reaction, such as  $\alpha$ -ketoglutarate  $\rightarrow$  succinate, with an inhibitor to block further oxidation of the product. In the early phases of this work malonic acid and several other substances were used in an attempt to achieve such conditions. Every substance tried so far seems to inhibit phosphorylation, although the effect is least marked with malonate. To avoid this inhibition the experiments reported here were done without any inhibitor, with the result that slightly higher P:O ratios were obtained (Table I). Similar conclusions as to the effect of inhibitors of phosphorylation have been reached by Cross *et al.* (4) and Lehninger (7). The presence of malonate in Ochoa's experiments with  $\alpha$ -ketoglutarate may have reduced the phosphorylation. The failure of most of the succinate to be further oxidized immediately when malonate is omitted must be due to the low substrate concentrations, for the same enzyme preparation rapidly oxidizes succinate added in higher concentrations. If succinate oxidation should occur, it would actually lower the P:O ratio, since unpublished data indicate a P:O ratio of 1.7 to 1.8 for succinate oxidation in this system.

The question as to whether changes due to the anaerobic reaction should be deducted is a debatable one. While there is the general tendency to assume that such anaerobic reactions would be less prominent in the vessels of the aerobic experiment, the possibility exists that the dismutation might be more prominent, due to release of more ammonia from the tissue under aerobic conditions. In any case we know that the dismutation results in esterification of only 0.9 molecule of phosphate for each 2 molecules of  $\alpha$ -ketoglutarate used.

Ochoa's P:O value of 3.0 for the complete oxidation of pyruvate to

carbon dioxide and water is based on directly observed values that were close to 2.0 plus carefully determined corrections for losses in side reactions. The suggestion of Ogston and Smithies (2) that values even as high as 2.0 are somewhat unlikely is based on several assumptions which appear to be unjustified. They assume that 16,700 calories are essential for the generation of every phosphate bond, when most of them may be generated at about a 12,000 to 13,000 calorie level. They also assume that only the oxidation-reduction potential below the level of cytochrome *c* can be used for generating energy-rich phosphate bonds. Finally, they challenge the validity of Ochoa's corrections for losses in side reactions. It seems certain that losses occurred in Ochoa's system, and that major corrections were justified.

The directly determined P:O ratios of 3.5 for  $\alpha$ -ketoglutarate oxidation reported here suggest that relatively small corrections for losses in side reactions would bring the value up to 4.0. Using 4.0 for  $\alpha$ -ketoglutarate and 2.0 for succinate, one might employ relative oxidation-reduction potential levels to estimate that the P:O ratio would be 4.0 for pyruvate  $\rightarrow$  active acetate, 3.0 for isocitrate  $\rightarrow$  oxalosuccinate, and 3.0 for malate  $\rightarrow$  oxalacetate. Thus, in the five oxidative steps in the oxidation of pyruvate, 16 energy-rich phosphate bonds would be generated for an average P:O of 3.2. This figure is not far from the corrected value reported by Ochoa. Lehninger (9) has recently reported data suggesting the value (corrected) of 3.0 for systems at the oxidation-reduction level of the  $\beta$ -hydroxybutyrate-acetoacetate system. Cross *et al.* (4) have reported at least one value over 3.0 for  $\alpha$ -ketoglutarate oxidation in indicating the range covered by their average of 2.4.

The point of origin of the high energy phosphate bonds during  $\alpha$ -ketoglutarate oxidation in general remains to be determined. If the value of 4.0 is accepted, the individual phosphorylations can tentatively be assigned to the general steps already known in the electron transfer systems somewhat as follows: (1) substrate oxidation; (2) oxidation of some co-enzyme at the diphosphopyridine-triphosphopyridine nucleotide level, although neither of these has been proved to participate in  $\alpha$ -ketoglutarate oxidation; (3) oxidation of a flavoprotein; (4) oxidation in the cytochrome region. Such an assignment of the phosphorylations is made on the basis of considerations of energy and *standard* oxidation-reduction potentials; *i.e.*, on the assumption that there are present approximately equal amounts of the oxidized and reduced components of each system. Only extreme divergence from standard conditions would upset these assignments.

#### SUMMARY

1. When  $\alpha$ -ketoglutarate is oxidized to succinate at 15° by washed rat liver particles supplemented with hexokinase and glucose, directly deter-

mined P:O ratios (inorganic phosphate removed to atoms of oxygen consumed) of 3.5 to 3.9 can be obtained.

2. When corrections are made for possible side reactions, the P:O ratios are still definitely over 3.0 (average 3.4 to 3.5), a fact which suggests that the actual value may be 4.0.

3. Use of incubation temperatures lower than those commonly used seems important for obtaining high P:O values consistently.

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# URIC ACID AND ALLANTOIN EXCRETION IN NORMAL AND TUMOR-BEARING MICE\*

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The nature of the action of chemical agents on neoplastic cells is receiving considerable attention at the present time. Kopac (1) reported that stilbamidine forms an insoluble complex with ribonucleate, liberating the protamine normally attached to the nucleic acid. Numerous studies with colchicine (2) suggest that this agent also interferes with the metabolism of nucleoprotein. Certain nitrogen mustards (3, 4) have been shown to have a selective action on lymphocytes, cells which contain high concentrations of nucleoproteins.

Krizek, Silverbach, and Schwartz (5) gave lethal doses of x-rays to dogs and observed no increase in uric acid or allantoin excretion. Since these animals were given total body radiation, the lymphoid tissues, which are large reservoirs of nucleoproteins, were included. This observation was somewhat unexpected, since in many ways the effects of x-rays on tissues resemble those of "chemomitotic poisons." This suggested that there might be some interference with nucleoprotein catabolism under the conditions of the study. It seemed desirable to conduct similar studies with chemical agents. Since large masses of lymphoid tissue can be obtained by employing animals bearing specific tumors, mice bearing the Gardner lymphosarcoma were selected for this investigation. This further permitted a comparison of the response of normal and malignant lymphoid tissue under identical conditions.

## EXPERIMENTAL

Pure strain and F<sub>1</sub> hybrid, C<sub>3</sub>H mice of both sexes were used in this investigation. The animals were maintained on Purina dog chow and given tap water *ad libitum*.

The Gardner lymphosarcoma, 6C<sub>3</sub>HED, was transplanted subcutaneously by trochar, the tissue being deposited half way between the fore and hind limbs on the right side of the animal. Therapy was begun on the tumor-bearing mice approximately 11 days after the transplantation, at a time when a mass was readily discernible and easily measured. The longi-

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tudinal and transverse diameters of each tumor were measured with calipers and the mean dimension (one-half the length plus the width) expressed in mm. At the end of the experimental period, the animals were sacrificed by severing the cervical cord, the spleens removed, cleaned, and weighed immediately.

The selected chemicals were administered, intraperitoneally, bis( $\beta$ -chloroethyl)amine (nitrogen mustard) as 0.1 per cent solution in saline and colchicine U. S. P. as 0.02 per cent solution in distilled water. The control animals received an equivalent amount of the appropriate solvent.

Uric acid in the urine was determined according to the method of Folin (6, 7). For blood uric acid Folin's revised micromethod was followed. Calculation of the amount of non-uric acid color in the urine after incubation with uricase (8) showed that 92 to 97.8 per cent of the amount obtained by the preceding method was true uric acid color. Allantoin in the urine was determined by the method of Young and Conway (9), and urine creatinine was determined according to the standard Folin procedure (10).

To obtain sufficient quantities of urine for study it was necessary to use that amount excreted by a group of ten animals. Urine samples were collected for a period of 3 hours, during which the animals had no access to food or water. This prevented any possible dilution of the sample or its contamination with food. The urine was removed from the collecting vessels at hourly intervals and frozen immediately. The completion of the collection period was 6 hours after therapy (average amount, approximately 1.5 cc.). 24 hour collections were not employed because of the difficulty of obtaining uncontaminated specimens. Since 3 hour urine specimens were used, uric acid and allantoin excretion over a 24 hour period could not be studied. The method of Forsham *et al.* (11), in which uric acid-creatinine ratios are employed, was adopted.

Blood was collected by decapitation 6 hours after the last treatment on the 3rd day of therapy.

#### DISCUSSION

A search of the literature failed to reveal any data on the excretion of uric acid or allantoin in mice. Since only the urine excreted in a 3 hour period could be employed, it was essential that the creatinine excretion remain constant and reproducible. In untreated groups of ten animals each, the urine creatinine values varied between 90 and 100 mg. per cent. In treated groups only occasional values were found as low as 80 mg. per cent, and only rarely did one exceed 100 mg. per cent.

Fig. 1 illustrates a typical experiment showing the uric acid and allantoin excretion in untreated tumor-bearing  $C_{3}H$  mice. Uric acid, allantoin, and creatinine were determined as mg. per 100 cc. of urine.

The uric acid-creatinine ratios are seen to increase steadily until they reach a plateau 6 or 7 days before death and remain about 100 per cent above the initial values. In contrast, the allantoin-creatinine ratios remain at normal levels until about 48 hours preceding death, at which time they become definitely elevated.

Fig. 2, A illustrates the effects of colchicine and nitrogen mustard on tumor growth and spleen weight. Colchicine in doses of 0.75 mg. per kilo daily for 3 days was found to have consistently striking effects in producing

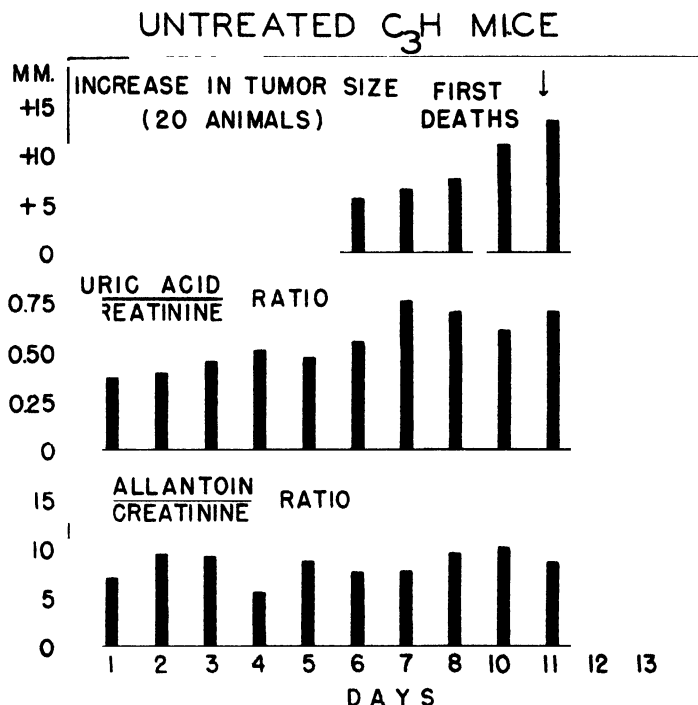


FIG. 1. Uric acid and allantoin excretion in untreated tumor-bearing mice, strain  $C_3H$ .

tumor regression. The group of mice receiving nitrogen mustard (4 mg. per kilo initially and 2 mg. per kilo per day thereafter) show some tumor regression and a definite reduction in the weight of the spleen. It is seen that an amount of colchicine which was sufficient to produce much more complete tumor regression than is seen with nitrogen mustard had little or no effect upon spleen weight. These findings after colchicine therapy are not in agreement with those of Leblond (12), who, using the rat as the experimental animal, found that colchicine produced a very definite reduction in the size of the spleen.

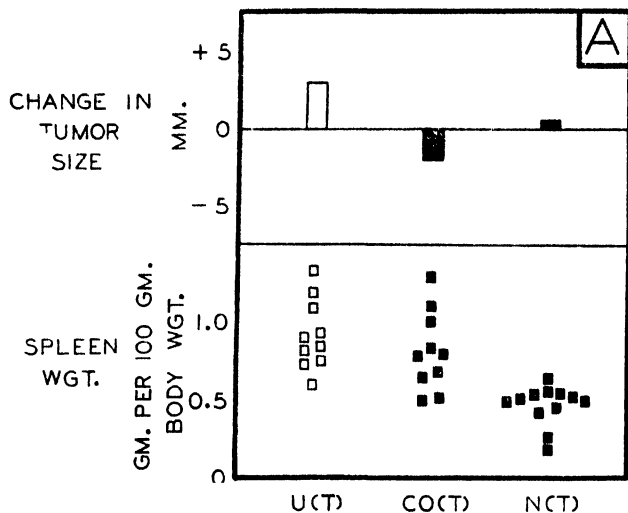


FIG. 2, A. Effect of colchicine and nitrogen mustard.  $C_3H$   $F_1$  hybrid mice bearing Gardner tumor. *U* = untreated, *CO* = colchicine-treated, *N* = nitrogen mustard-treated, (*T*) = animals bearing tumors.

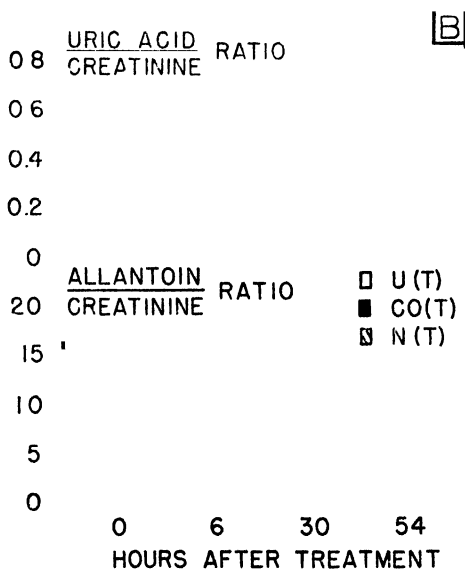


FIG. 2, B. Conditions as for Fig. 2, A

Fig. 2, B shows the uric acid-creatinine and allantoin-creatinine ratios obtained from a study of the urine of these same animals. Both colchicine and nitrogen mustard caused an elevation of the uric acid-creatinine ratio,

but the colchicine effect was more pronounced. The allantoin-creatinine ratios were rather variable, but remained within the normal range.

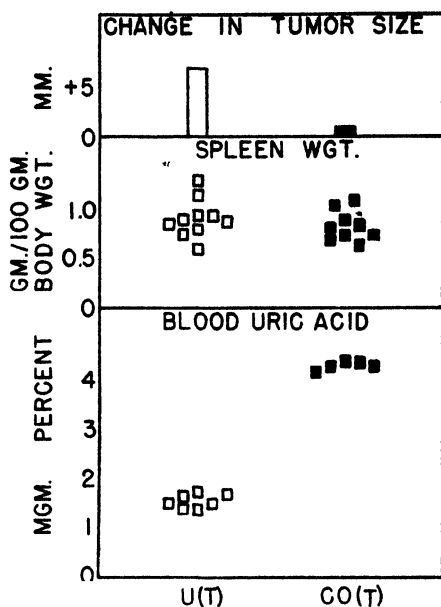


FIG. 3.  $C_4H F_1$  hybrid tumor-bearing mice treated with colchicine. Animals sacrificed 54 hours after initial therapy.  $U(T)$  = untreated,  $CO(T)$  = colchicine treated.

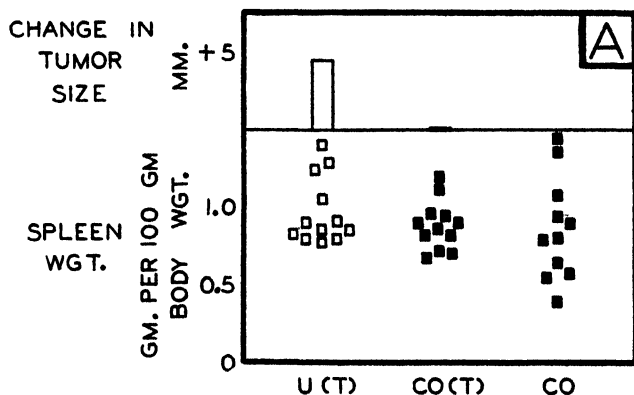


FIG. 4, A. Effect of colchicine.  $C_4H F_1$  mice.  $U$  = untreated,  $CO$  = colchicine-treated,  $(T)$  = animals bearing tumors.

Blood uric acid was determined in colchicine-treated tumor-bearing mice. Fig. 3 shows a significant increase in these values under conditions which

resulted in marked tumor regression. However, such striking differences were not uniformly obtained.

Another experiment was designed to compare the effects of colchicine upon tumor-bearing mice with its action in animals free of malignant tissue. Untreated tumor animals were also used for comparison. Again it is seen in Fig. 4, *A* that, although colchicine caused a marked tumor regression, it produced very little if any change in spleen weight of either tumor-bearing mice or the normal controls. Fig. 4, *B* shows the characteristic increase in uric acid-creatinine ratios obtained when tumor-bearing mice are treated with a dose of colchicine sufficient to cause definite tumor regression. The

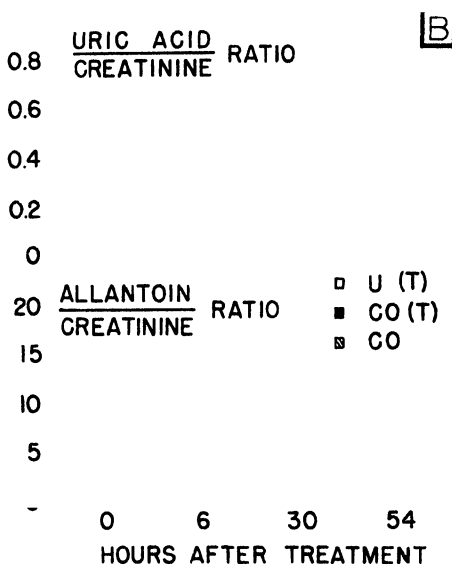


FIG. 4, *B*. Conditions as for Fig. 4, *A*

non-tumor animals with colchicine showed about the same increase in uric acid-creatinine ratios as was demonstrated in the untreated tumor-bearing mice.

#### SUMMARY

Uric acid and allantoin excretion has been determined in both tumor-bearing and non-tumor-bearing mice treated with colchicine. Similar studies have been made with nitrogen mustard in tumor-bearing mice. In every instance uric acid excretion was increased, with little or no change in the allantoin excretion.

The increase in uric acid excreted was greatest in the colchicine-treated tumor-bearing mice.

The fact that the uric acid excretion was increased and the allantoin excretion was not reduced after the administration of nitrogen mustard or colchicine suggests that there is no significant alteration in the normal mechanism of purine catabolism.

In mice the response of malignant lymphoid tissue to colchicine is more striking than that of the lymphoid tissue of the normal spleen. As reduction in weight of the spleen in the mouse is consistently seen when "alarming" stimuli are employed (13), it is suggested that the increase in uric acid excretion and tumor regression observed are due to a specific action of the chemical on the lymphoid tissue.

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# A NEW AND CONVENIENT SYNTHESIS OF 4-AMINO-5-IMIDAZOLECARBOXAMIDE

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An amine formed by *Escherichia coli* during sulfonamide bacteriostasis (1) has been identified as 4-amino-5-imidazolecarboxamide (V) (2). Since sulfonamide inhibition apparently blocks the biosynthesis of purines, the suggestion has been made (2) that the aminoimidazole carboxamide (V) is a precursor of purines and accumulates in the medium due to a blocked reaction involving the insertion of the single carbon atom necessary for the completion of the purine ring system. Further study of the possible metabolic rôle of this amine would be facilitated by an adequate source of material. A synthesis described by Windaus and Langenbeck (3) starting with methylglyoxal was found to provide an over-all yield of 1 per cent of the desired base. Therefore, an alternate method of preparation was undertaken. Making use of a new imidazole synthesis in which a formamidoacetamide is cyclized to a 4-aminoimidazole, we have been able to prepare 4-amino-5-imidazolecarboxamide in an over-all yield of 30 per cent, starting with ethyl cyanoacetate.

The imino ether of ethyl cyanoacetate (I) was treated with alcoholic ammonia, simultaneous introduction of the amidine and amide groups taking place. The resultant malonamamidine (II) was coupled with benzenediazonium chloride to yield the phenylazo derivative (III). When the azo compound was reduced with zinc dust in 98 per cent formic acid, the formamido derivative of malonamamidine (IV) was obtained. The formyl group in this compound exhibited the expected lability to acid, undergoing ready deformylation to the amino amidine (VII). As the free base, the formamido amidine (IV) hydrolyzed in aqueous solution to formamido-malonamide (VI).

Ring closure of the formamido amidine (IV) to 4-amino-5-imidazolecarboxamide (V) was achieved most conveniently merely by melting the formamido compound as the hydrochloride. Eventually the mass crystallized as amino imidazole hydrochloride. At 170°, for example, the thermal cyclization was complete in 10 minutes, yielding 89 per cent of V after one recrystallization. Although the base hydrochloride was described by Stetten and Fox (1) as having a melting point of 210–215°, the hydro-

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Pinner mentioned the synthesis of this amidine (5) without giving details and recorded the melting point as 150°.

*Phenylazomalonamidine Hydrochloride (III)*—Aniline (10.5 ml.) dissolved in 6 N hydrochloric acid (65 ml.) was diazotized below 5° by the gradual addition of a solution of sodium nitrite (8.5 gm.) in water (50 ml.). 5 minutes after the addition was complete, excess nitrous acid was decomposed with urea. The diazonium solution was then poured into a solution of the amidine (II) (15.5 gm.) in water (75 ml.) and the pH of the resultant mixture brought to about 4 by the addition of a concentrated aqueous sodium acetate solution. Yellow needles of azo compound began to separate. The reaction was allowed to proceed at room temperature for several hours, then left overnight in the ice box to complete crystallization. The filtered product was washed with a small amount of water and desiccated *in vacuo*, yielding 24.2 gm., 90 per cent, m.p. 196–198°. Recrystallized from alcohol and ether, a sample was obtained which melted at 199–200°.

$C_9H_{12}ON_4Cl$ . Calculated, Cl<sup>-</sup> 14.67, N 28.98; found, Cl<sup>-</sup> 14.49, N 28.74

*Formamidomalonamidine Hydrochloride (IV)*—Phenylazomalonamidine hydrochloride (25 gm.) was added in portions during 1 hour to a suspension of zinc dust (50 gm.) in 98 per cent formic acid (275 ml.). The mixture was agitated occasionally, but no attempt was made to dissipate the heat of reaction. After the addition was complete, the suspension was warmed on a hot plate until the residual azo color was discharged, then filtered. The filtered material was washed with formic acid and the combined filtrates concentrated to a syrup *in vacuo*. The concentration was repeated with several additions of water until the odor of formic acid was no longer evident, the temperature of the material being kept below 50° during the process. The residue was taken up in water (100 ml.) and freed of zinc by means of hydrogen sulfide. The filtrate from the zinc sulfide was now taken to a syrup *in vacuo* with repeated additions of absolute alcohol to remove water. To separate the product from the formanilide formed in the reaction, the residue was dissolved in methanol and treated dropwise with methanolic HCl until a strongly acidic reaction was shown on addition of a drop to moistened indicator paper. The addition, without delay, of a large volume of anhydrous ether precipitated the product as a gum from which the ethereal supernatant containing formanilide was shortly decanted. A few drops of water added to the product induced crystallization. The mass was broken up with 95 per cent ethanol added gradually, followed by anhydrous ether to complete crystallization. The yield was 12.3 gm. or 63 per cent calculated as the hemihydrate; m.p. 93–95°. While the product could be recrystallized in well formed crystals from aqueous ethanol

by the addition of ether, the melting points after successive crystallizations varied in either direction, due to the instability of the material in this treatment. The product obtained on the first crystallization was generally used in the imidazole cyclization without further attempt at purification. For analysis, a recrystallized sample, m.p. 95–96°, was dried over  $P_2O_5$  at 56°.

$C_4H_5O_2N_4Cl \cdot \frac{1}{2}H_2O$ . Calculated, Cl- 18.70, N 29.56; found, Cl- 18.37, N 29.51

Formamidomalonamamidine did not couple with diazotized sulfanilic acid, but a strongly alkaline solution warmed in a water bath for a few minutes did give an orange color in the coupling reaction. Such alkali-treated material also could be diazotized and coupled, as in the Bratton-Marshall procedure. Some formation of amino imidazole apparently takes place in alkaline solution. However, a preparative conversion of the formamido compound to the imidazole cannot be achieved under these conditions due to extensive hydrolysis of the amidine group with the formation of formamidomalonamide (VI) as shown by the following experiment.

Formamidomalonamamidine hydrochloride (0.425 gm.) was converted to the free base by addition of 0.1 N NaOH (25 ml.) and the solution was refluxed for 1½ hours, whereupon ammonia was evolved. The solution was taken to dryness and the residue recrystallized from water. The crystals obtained, m.p. 203–205°, did not depress the melting point of formamidomalonamide (6). A yield of 52 per cent was obtained.

*Aminomalonamamidine Dihydrochloride (VII)*—The formamido compound (3.2 gm.) was refluxed with methanolic HCl (75 ml.) plus sufficient water added dropwise to the boiling suspension to bring about solution. After 10 minutes, the solution was taken to dryness *in vacuo*. The residue was thinned with a few drops of water and crystallized by the slow addition of absolute alcohol and ether. The product was obtained as colorless crystals, 2.5 gm., m.p. 208–209°, in a yield of 78 per cent. A sample recrystallized by the above method melted at 209–210°.

$C_5H_{10}ON_4Cl_2$ . Calculated, Cl- 37.55, N 29.62; found, Cl- 37.22, N 29.66

*4-Amino-5-imidazolecarboxamide Hydrochloride (V)*—Formamidomalonamamidine hydrochloride (2.5 gm.) was heated in a flask placed in an oil bath whose temperature was held at about 170°. The amidine melted and, within 10 minutes, most of the mass crystallized as the aminoimidazolecarboxamide. The flask was cooled and the product recrystallized from a concentrated aqueous solution by the addition of ethanol followed by ether, yielding 1.9 gm., 89 per cent, melting with decomposition at 255–256°. The melting point was unchanged by further recrystallization.

$C_4H_7ON_4Cl$ . Calculated. Cl- 21.81, C 29.54, H 3.72  
Found. " 21.36, " 29.39, " 4.13

When the cyclization was carried out at 130°, crystallization of the molten material did not take place until it had been heated for 20 minutes. In this run a gentle vacuum was applied intermittently to facilitate the removal of water formed in the reaction.

4-Amino-5-imidazolecarboxamide was also prepared by the method of Windaus and Langenbeck (3) with modifications suggested by Allsebrook, Gulland, and Story (7). The hydrochloride obtained melted at 255–256° with decomposition and this melting point was not depressed by admixture with the hydrochloride obtained as described above. In addition, the corresponding free bases melted at 168–169° and yielded picrates melting at 237–238°; in each case mixed melting points gave no depression.

#### SUMMARY

A new method has been described for the synthesis of 4-amino-5-imidazolecarboxamide, an amine accumulated by *Escherichia coli* during sulfonamide bacteriostasis. This method, which is superior in ease of operation and yield to the available method, starts with ethyl cyanoacetate which is converted in five steps to the desired base in an over-all yield of 30 per cent.

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# THE CHEMISTRY OF CIRCULIN;\* CHROMATOGRAPHIC ISOLATION OF THE AMINO ACID CONSTITUENTS WITH POWDERED CELLULOSE

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In May, 1948, Murray and Tetrault (1) announced the discovery of an antibiotic more active against Gram-negative than Gram-positive organisms. Murray, Tetrault, *et al.* (2) later published an assay method and identified the organism as a non-hemolytic strain of *Bacillus circulans*. Peterson *et al.* (2), collaborating with the latter workers, described the isolation and purification of circulin. The similarity of this antibiotic to the known polymyxins was suggested, although it appeared to be different from polymyxin D on the basis of its response to crude trypsin and its bacterial spectrum.

Circulin was prepared by the method of Peterson *et al.* (2). Briefly, this process consisted of adsorption of the antibiotic from the culture filtrate on activated carbon (Dareo G-60) and elution with aqueous acid tertiary butanol solutions. On further chromatography of circulin sulfate by methods previously described by Peterson *et al.* (2), a purified compound has now been obtained. Elemental analysis and analytical data are in agreement with a cyclic compound whose empirical formula is  $(C_{38}H_{74}O_9N_{12})_n$ . Circulin sulfate is a white amorphous solid; m.p. 226–228°,  $[\alpha]_D^{25} = -61.6^\circ$  ( $c = 1.25$  in water). Circulin sulfate is very soluble in water, less soluble in the lower alcohols, and insoluble in non-polar solvents.

Circulin is a basic polypeptide containing leucine, threonine,  $\alpha, \gamma$ -diaminobutyric acid, and an optically active isomer of pelargonic acid. The foregoing studies are chiefly concerned with the isolation, composition, and structure of the polypeptide, and comparison is made with the polymyxin type of antibiotics previously described by the American and British workers (3, 4). Studies on the toxicity of circulin will be reported by Vander Brook (5). In general, circulin is similar to polymyxin A (aerosporin) described by Brownlee *et al.* (6).

## EXPERIMENTAL

In the following experimental work melting points were determined by use of capillary tubes in a silicone bath unless otherwise specified. All

\* In a recent note to the *Journal of Bacteriology* (in press) the name circulin was withdrawn in favor of polypeptin for an antibiotic described by Charlotte McLeod (*J. Bact.*, **56**, 749 (1948)).



specific rotations were determined in a 1 dm. micro tube. Hydrolyses were accomplished in a closed vessel at 120° for 7 hours with 3 N hydrochloric acid. The reaction between circulin and 2,4-dinitrofluorobenzene<sup>1</sup> was performed according to the technique of Sanger (8). This latter compound has been referred to as DNP-circulin throughout this report.

17 gm. of circulin sulfate (5500 to 6500 units per mg.) were rechromatographed over Darco G-60 as previously described (2). The combined fractions (15.62 gm.) assayed 5500 to 6500 units per mg. The potency of no single fraction was greater than that of the combined fractions. The purified product as prepared by this method was soluble in water to an extent greater than 40 per cent, less soluble in the lower alcohols, insoluble in the water-immiscible solvents and acetone. Paper chromatography with 25 per cent acetic acid, 50 per cent *n*-BuOH, and 25 per cent water (by volume) as the developing solution indicated the presence of only one antibiotic. This product had a melting point of 226–228°, and a specific rotation of  $-61.6^\circ$  ( $c = 1.25$  in  $H_2O$ ). The amino nitrogen before hydrolysis was 7.52 per cent, after hydrolysis 15.85 per cent. The ninhydrin- $CO_2$  (Van Slyke) test was negative. The ash was negligible. Detailed analytical data are given in a later section.

*Preparation of Circulin Picrate*—400 mg. of circulin sulfate, assaying 6000 units per mg., were dissolved in 9 ml. of water. To this solution were added 500 mg. of picric acid in 5 ml. of acetone and the final volume brought to 30 ml. with water. The yellow precipitate was filtered and washed three times with 2 ml. portions of water. The dried picrate weighed 555 mg. (recovery based on bioassay 91 per cent) and assayed 3200 to 3600 units per mg. in 50:50 acetone-buffer solution; m.p. 160–168°, decomposition.

*Preparation of Circulin Helianthate*—200 mg. of circulin sulfate (6000 units per mg.) were dissolved in 2 ml. of water. After complete precipitation by the addition of 200 mg. of methyl orange in 35 ml. of water, the circulin helianthate was collected by filtration. The precipitate was washed with water, and after drying 275 mg. (recovery based on bioassay, 70 per cent) were obtained, assaying 2600 to 2800 units per mg.; m.p. 218–222°, decomposition.

*Preparation of Circulin Reineckate*—200 mg. of circulin sulfate (6000 units per mg.) were dissolved in 2 ml. of water and 170 mg. of ammonium reineckate in 10 ml. of water were added. The precipitate was filtered and washed with 1 ml. portions of water. The dried product weighed 172 mg. (recovery based on bioassay, 33 per cent) and assayed 2400 to 3300 units per mg.; it darkened at 185–195° without melting.

*Preparation of Circulin Hydrochloride from Circulin Picrate*—To 270

<sup>1</sup> Prepared by Dr. J. W. Hinman in these laboratories by the method of Sanger (7).

mg. of circulin picrate (3300 units per mg.) in 6 ml. of acetone, gaseous HCl was added until no further precipitation occurred. The precipitate of circulin hydrochloride was filtered, washed with acetone, and dried from the frozen state. 109 mg. of powder (recovery based on bioassay, 79 per cent) were obtained, assaying 6500 units per mg.; m.p. 226–230°, decomposition.

By substituting concentrated sulfuric acid for hydrochloric acid, circulin sulfate was prepared.

*Preparation of Formaldehyde Derivative of Circulin*—To 1.0 gm. of circulin hydrochloride (6500 units per mg.) in 4.0 ml. of water, 3.0 ml. of formalin were added. After 30 minutes 0.14 gm. of sodium hydroxide in the form of a saturated solution was added. A white water-insoluble precipitate was collected and washed thoroughly with water. The dried product weighed 750 mg. (recovery based on bioassay, 79 per cent) and assayed 6300 units per mg.; m.p. 242–250°, decomposition.

*Preparation of 2,4-Dinitrophenyl Derivative of Circulin*—To 105 mg. of circulin hydrochloride (6500 units per mg.) in 3 ml. of water, 100 mg. of sodium bicarbonate were added. After shaking the above solution with 112 mg. of 2,4-dinitrofluorobenzene for 2 hours at 37°, a yellow, water-insoluble precipitate formed. The precipitate was washed thoroughly with ether and water and upon drying yielded 170 mg. of the inactive derivative; m.p. 158–163°, with softening at 135–140°.

*Preparation of Acetyl Derivative of Circulin*—To 250 mg. of circulin hydrochloride (6300 units per mg.) dissolved in 5 ml. of water, 1 per cent sodium carbonate solution was added until pH 8.5 was attained. Then 0.7 ml. of acetic anhydride was added. After 5 minutes stirring a water-insoluble precipitate appeared. The precipitate was washed thoroughly with water, giving 280 mg. of an inactive dry solid which darkened at 235–245° without melting.

*Van Slyke Reaction*—100 mg. of circulin (6000 units per mg.) were dissolved in a mixture of 3 ml. of 30 per cent sodium nitrite and 1 ml. of glacial acetic acid. This solution was stirred for 5 minutes and evaporated to dryness under a vacuum. The solids were dissolved in water and assayed 200 to 300 units per mg., based on the starting material. No attempt was made to isolate the derivative.

*Combining Weights*—Combining weights were determined in the following manner. To a known amount of the antibiotic in solution, a known weight of the precipitating agent was added in excess. Based on the dry weight of the precipitate, the weight of the excess reagent recovered, and the assays of each fraction, the combining weights were determined. They were found to be 320 for the picrate, 290 for the sulfate, and 300 for the hydrochloride.

*Microbiological Studies*—These studies were only roughly quantitative

(30 to 40 per cent error) but served as a guide to later isolation work. An acid hydrolysate of circulin under these conditions showed the presence of 8 to 9 per cent threonine and 1 per cent leucine. However, when the hydrolysate was racemized with sodium hydroxide, the microbiological assays showed 4.2 to 4.6 per cent threonine and 3.5 to 4.0 per cent leucine. These results indicated roughly the presence of 8 to 9 per cent L-threonine and 7 to 8 per cent D-leucine.

*Degradation Studies; Crystallization and Identification of L- $\alpha$ , $\gamma$ -Diaminobutyric Acid*—5 gm. of circulin hydrochloride, assaying 6500 units per mg., were hydrolyzed with hydrochloric acid. An ether extract of the acid hydrolysate was retained for studies on the identification of the fatty acid fraction. After removal of the hydrochloric acid by the usual method of evaporation, the concentrate was dissolved in 90 ml. of warm 85 per cent ethanol. On cooling, a mass of plate-like crystals formed. These were recrystallized twice, giving chromatographically pure  $\alpha$ , $\gamma$ -diaminobutyric acid monohydrochloride; m.p. 228–230°,  $[\alpha]_D^{25} = +8^\circ$  ( $c = 1.0$  in water).

*Analysis*— $C_4H_{10}O_2N_2 \cdot HCl$  (154.6)

Calculated. C 31.06, H 6.48, N 18.11, Cl 22.95

Found. " 30.85, " 6.80, " 17.91, " 23.00

In order to determine the configuration of the  $\alpha$ , $\gamma$ -diaminobutyric acid 200 mg. of the naturally occurring compound were converted to the oxalate by the method of Adamson (9). A salt was obtained with a melting point of 167–170° (capillary), 211–213° (corrected melting point on a Kofler micro block), and a specific rotation of +8.5° ( $c = 4.0$  in water for the anhydrous salt). Adamson (9) records a melting point of 211–215° and a specific rotation of +8.3° ( $c = 3.98$  in  $H_2O$  for the anhydrous salt) for the oxalate of L- $\alpha$ , $\gamma$ -diaminobutyric acid.

As this paper was being written a report appeared by Catch, Jones, and Wilkinson (10) giving a fractional crystallization method for separating  $\alpha$ , $\gamma$ -diaminobutyric acid from acid hydrolysates of polymyxins A and B. These authors also obtained pure crystalline D-leucine and L-threonine from polymyxin A.

The fatty acid fraction, which represented 10 per cent of the original polypeptide, was obtained by extraction of the acid hydrolysate at pH 1.2 with ether. The ether was then extracted with sodium bicarbonate solution. The bicarbonate solution was acidified to pH 1.2 and extracted with ether. After washing with water, the ether solution was evaporated and the fatty acid dried under a vacuum. The purified product gave a neutralization equivalent of 160 (calculated 158) and a specific rotation of +7.5° ( $c = 1.41$  in ether).

To 100 mg. of the fatty acid 0.5 ml. of thionyl chloride was added. After

refluxing for  $\frac{1}{2}$  hour, 3 ml. of ice-cold concentrated ammonium hydroxide were added and the amide formed was dissolved and crystallized from 30 per cent ethanol. After recrystallization the dried platelets melted at  $94.5^\circ$ , uncorrected, and when mixed with known pelargonic acid amide (m.p.  $94^\circ$ ) a depression of  $4^\circ$  resulted. The infra-red spectrum of the unknown amide differs only slightly from that of known pelargonic acid amide and can be interpreted as signifying a small structural difference. This confirms the fact that we are probably dealing with a branched chain compound isomeric with pelargonic acid as indicated by the above specific rotation. Catch, Jones, and Wilkinson (10) have isolated a fatty acid with similar properties from polymyxin A.

*Analysis of Fatty Acid Amide—*

Known pelargonic acid amide. Calculated. C 68.85, H 12.18, N 8.91

$C_9H_{19}NO$  (157)

Unknown.

Found. " 68.41, " 11.88, " 8.99

*Separation of L- $\alpha$ , $\gamma$ -Diaminobutyric Acid, D-Leucine, and L-Threonine by Partition Chromatography with Powdered Cellulose—*Since the separation of the amino acids of circulin presented a rather difficult problem from the standpoint of fractional crystallization, the possibility of using paper chromatography on a larger scale occurred to the authors. Consequently, 10 gm. of a fine grade of cellulose (Solka Floc<sup>2</sup>) was stirred with 50 ml. of a solution composed of 25 per cent glacial acetic acid, 50 per cent *n*-butanol, and 25 per cent water as used in paper strip chromatography throughout this report. This slurry was stirred  $\frac{1}{2}$  hour and filtered, the cellulose resuspended in the solvent, and this operation repeated. The slurry was then placed in a column 12 mm. in diameter and packing was accomplished by applying an air pressure of 5 pounds. The height of the packed cellulose column was 37 cm. Three Vml. of the solvent were passed over the column. (Vml. is defined as the volume of solvent which is required to wet completely the column of adsorbent.) At this point, 25 mg. of leucine, 25 mg. of threonine, and 50 mg. of  $\alpha$ , $\gamma$ -diaminobutyric acid were dissolved in 3 ml. of the solvent and placed on the column. Chromatography then proceeded and samples of 1.0 ml. each were collected at a rate of 1.0 to 2.0 ml. per hour. Under these conditions a favorable separation of the amino acids was obtained, as shown in Fig. 1. The fractions were tested for amino acid composition by ascending paper strip chromatography. In order to reduce the number of tests required for the paper strip chromatograms, 0.001 ml. of each fraction was dried on a filter paper, sprayed with ninhydrin, and heated. If no spot appeared, it was not nec-

<sup>2</sup> Solka Floc BW-200 obtained from the Brown Company, 500 Fifth Avenue, New York, 18.

essary then to run a paper strip chromatogram. The samples containing the respective amino acids were weighed and pooled. After removal of the solvents *in vacuo*, the amino acids were crystallized from ethanol. The total recoveries based on weight were leucine 88 per cent, threonine 84 per cent, and  $\alpha, \gamma$ -diaminobutyric acid 92 per cent.

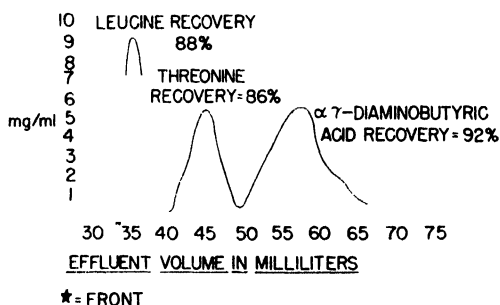


FIG. 1. Separation of a known mixture of leucine (25 mg.), threonine (25 mg.), and  $\alpha, \gamma$ -diaminobutyric acid (50 mg.) by cellulose chromatography.

TABLE I  
*Analysis of Crystalline Amino Acids Obtained from Circulin*

Amino acid	Yield	M.p	$[\alpha]_D^{25}$	C	H	N	Cl
	mg.	°C.	degrees	per cent	per cent	per cent	per cent
L- $\alpha, \gamma$ -Diaminobutyric acid $C_4H_{10}O_2N_2 \cdot HCl$ (154.6)	1500	228	+9.2 (c 1.0)*	30.75 31.06	6.79 6.48	17.85 18.11	23.15 22.95†
D-Leucine $C_6H_{13}O_2N$ (131.1)	80	233	+10.1 (" 2.0)*	55.30 54.92	9.75 9.99	10.67 10.68	†
L-Threonine $C_4H_9O_3N$ (119)	54	285	-26.0 (" 2.0)*	39.95 40.34	7.64 7.54	11.71 11.76	†

\* In water.

† Calculated values.

In one experiment a column was prepared with 50 gm. of cellulose in a tube of 17 mm. diameter. The height of the packed cellulose column was 60 cm. 5.0 gm. of an acid hydrolysate freed of fatty acid were put over this column at a rate of 3 to 4 ml. per hour. 2 ml. fractions were collected. From the best fractions of each amino acid the crystalline material was prepared. Recrystallization from ethanol gave pure D-leucine, L-threonine, and L- $\alpha, \gamma$ -diaminobutyric acid monohydrochloride. The analytical data for these amino acids are listed in Table I.

An attempt was then made to obtain a clean cut separation of the amino acids from 300 mg. of hydrolyzed circulin. The fatty acid-free hydrolysate

was placed over a column 17 mm. in diameter, containing 30 gm. of cellulose. The packed cellulose column was 48 cm. in height and the conditions employed were the same as those used in separation of the known amino acids. Fig. 2 demonstrates that separation was satisfactorily accomplished. From the fractions obtained it was possible to crystallize and recrystallize D-leucine (10 mg. yield), L- $\alpha$ , $\gamma$ -diaminobutyric acid monohydrochloride (80 mg. yield), and L-threonine (12 mg. yield). The results shown in Fig. 2 demonstrate that circulin contains approximately 10 per cent leucine, 12 per cent threonine, and 55 to 65 per cent  $\alpha$ , $\gamma$ -diaminobutyric acid. By following the optical rotation of each sample of the  $\alpha$ , $\gamma$ -diaminobutyric acid, it is seen that the first break in the curve is apparently due to the dihydrochloride, while the remaining samples are

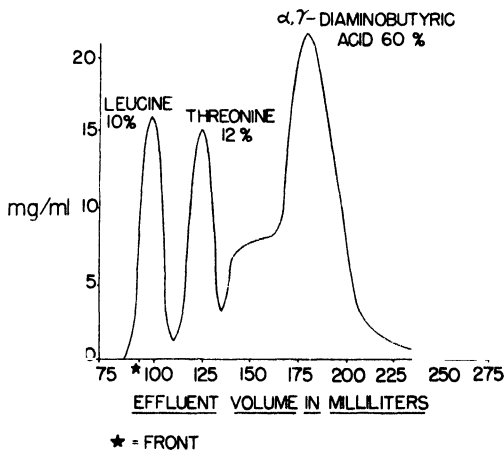


FIG. 2 Separation of the amino acids of circulin by cellulose chromatography

chiefly the monohydrochloride. No DL form was found in any of these fractions.

*Free Amino Group of Circulin* -The 2,4-dinitrophenyl derivative of circulin (DNP-circulin) was prepared according to the method of Sanger (8). This derivative of circulin was hydrolyzed with hydrochloric acid and compared by ascending paper strip chromatography to an acid hydrolysate of circulin hydrochloride, and to  $\alpha$ -amino- $\gamma$ -(2,4-dinitroanilino)-butyric acid<sup>3</sup> prepared by the method of Sanger (7). The hydrolyzed DNP-circulin produced only one new spot on the chromatogram and this spot was identical with one produced by  $\alpha$ -amino- $\gamma$ -(2,4-dinitroanilino)-butyric acid. Simultaneously with the production of the new spot, the

<sup>3</sup> Prepared and supplied by Dr. R. G. Shepherd, American Cyanamid Company, Stamford, Connecticut.

intensity of the  $\alpha, \gamma$ -diaminobutyric acid spot diminished. These results indicated that the  $\gamma$ -amino group of  $\alpha, \gamma$ -diaminobutyric acid contributed the free amino groups in the circulin molecule. However, at least part of the  $\gamma$ -amino groups either does not react with 2,4-dinitrofluorobenzene or is released on acid hydrolysis. The chromatogram is shown in Fig. 3. Ninhydrin (0.25 per cent) was used to develop the color. The  $R_F$  value for  $\alpha$ -amino- $\gamma$ -(2,4-dinitroanilino)-butyric acid<sup>3</sup> was 0.68 to 0.72 and was identical with that of the new spot. A faint spot occurring just above

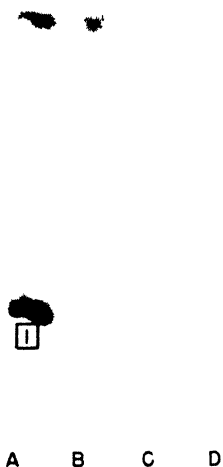


FIG. 3 Amino acid composition and free amino group of circulin. A, known mixture of  $\alpha, \gamma$ -diaminobutyric acid (1), threonine (2), and leucine (3); B, acid-hydrolyzed circulin; C, acid-hydrolyzed 2,4 dinitrofluorophenylcirculin; D,  $\alpha$ -amino- $\gamma$ -(2,4 dinitroanilino)-butyric acid

threonine in hydrolyzed circulin and DNP-circulin was interpreted as a small amount of an impurity.

**Titration Curve**<sup>1</sup>—One sample of circulin was titrated into the alkaline range with sodium hydroxide, another sample with hydrochloric acid. The graph in Fig. 4 shows the experimental data plotted as the difference between the sample titration and the blank titration. The difference has been calculated and plotted on an equivalence basis for a molecular weight of 625. The theoretical curve is plotted for a substance having titratable groups with  $pK'a$  at about 8.2 and 1.6. It can be seen that the experi-

<sup>4</sup> The titration curve with its interpretation was kindly supplied by Mr. T. V. Parke, Eli Lilly and Company, Indianapolis 6.

mental data fit the theoretical curve quite well between pH 3.0 and 8.2. The divergence of the experimental data above pH 8.2 suggests the existence of another basic group with  $pK'a$  at about 9.5. The group titrating at  $pK'a$  1.6 is only suggested by the data. It is certain, however, that there is no group having a  $pK'a$  above 1.6. These studies then indicated

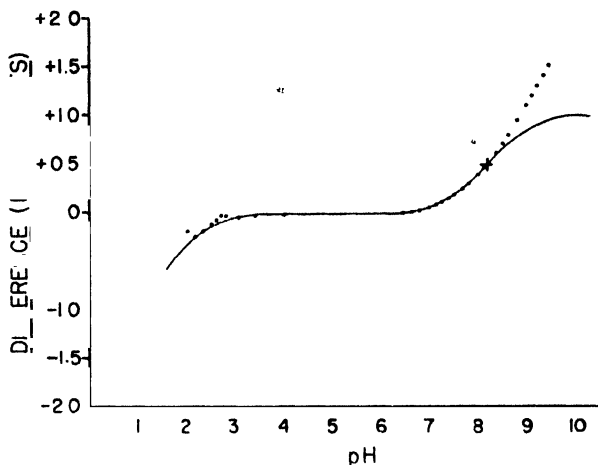


FIG. 4. Titration curve of circulin. Solid curve, theoretical curve for substance with groups at  $pK'a$  about 8.2 and 1.6; dotted curve, experimental points for circulin hydrochloride.

TABLE II  
*Analytical Data of Circulin Sulfate*

Element	Calculated	Found
	<i>per cent</i>	<i>per cent</i>
C . . . . .	44.55	44.60
H . . . . .	7.47	7.75
N . . . . .	15.99	16.08
SO <sub>4</sub> . . . . .	22.36	21.30

the presence of two basic groups of different strengths and the absence of free carboxyl groups above pH 1.7

On the basis of all these results it appears that circulin should contain 1 molecule each of D-leucine and L-threonine, and an isomer of pelargonic acid, together with 5 molecules of L- $\alpha,\gamma$ -diaminobutyric acid, linked together in a cyclic structure. The data in Table II support this belief. Accordingly the formula for the sulfate would be  $C_{39}H_{78}O_9N_{12} \cdot 2\frac{1}{2}H_2SO_4$ . The analytical figures are in excellent agreement with the theoretical except for the sulfate.



*Comparative Potencies of Circulin with Polymyxins*—Table III gives the relative potencies of what are believed to be the pure compounds, based on the standard plate assay method of circulin (2).

*Stability Studies*—Circulin sulfate in aqueous solution (2 mg. per ml.) at pH 8.0 and 37° for 16 days retained 90 per cent of the original activity. A sterile solution (10 mg. per ml.) kept at pH 6.5 and room temperature for 30 days lost 15 per cent of its activity. When aqueous solutions at pH 2.0 to 6.5 were autoclaved for 15 minutes, no significant loss in activity

TABLE III  
*Comparative Potencies of Circulin and Polymyxins*

Antibiotic	Circulin
	<i>units per mg</i>
Circulin sulfate . . . . .	6,300
Polymyxin A hydrochloride . . . . .	11,000
“ B “ . . . . .	6,700
“ D “ . . . . .	15,000

TABLE IV  
*Chemical Composition of Circulin and Polymyxins*

Constituent	Antibiotic*					
	Circulin	Polymyxin				
		A	B	C	D	E
D-Leucine . . . . .	+	+	+	—	+	+
L-Phenylalanine . . . . .	—	—	+	+	—	—
L-Threonine . . . . .	+	+	+	+	+	+
D-Serine . . . . .	—	—	—	—	+	—
$\alpha, \gamma$ -Diaminobutyric acid	+	+	+	+	+	+
Pelargonic acid type . . . . .	+	+	+	+	+	+

\* Jones (4).

was noted. Dried circulin sulfate stored for 5 months at 4°, 23°, and 37° suffered no loss in potency.

The chemical composition of circulin compared to the polymyxins, as reported by Jones (4), is given in Table IV. It can be seen that circulin has the same amino acid composition qualitatively as polymyxins A and E, but differs in this respect from polymyxins B, C, and D. On the basis of chemical composition and lipase studies which follow, it was possible to differentiate circulin from polymyxins A, B, C, and D.

*Effect of Trypsin and Lipase upon Circulin and Polymyxins A, B, and D*—The chemical composition of circulin places it in the class of the polymyxins (Table IV). Since these antibiotics are all polypeptides, it is of

interest to determine the effect of proteolytic enzymes. By such studies it was possible to differentiate circulin from polymyxin A, which has qualitatively the same amino acid composition. Stansly and Ananenko (11) have shown that polymyxin D is resistant to pepsin, "trypsin," pancreatin, and erepsin. Brownlee<sup>5</sup> has indicated that crude trypsin does not affect polymyxins A, B, and C.

When circulin sulfate was incubated with a 1 per cent crude trypsin<sup>6</sup> preparation, tested for proteolytic activity by the Mett method (12) at pH 7.8 to 8.0, approximately 80 per cent was inactivated in 10 days at 37°. Appropriate corrections were made with controls of boiled inactive enzyme.

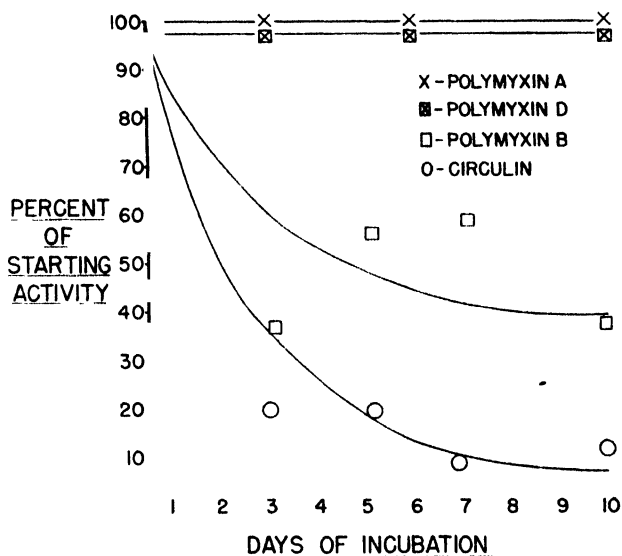


FIG. 5. Effect of lipase on circulin and polymyxins A, B, and D

Polymyxins A and D were unaffected by crude trypsin, while polymyxin B was inactivated at approximately the same rate as circulin. These results indicated that our crude trypsin was different from that used by Brownlee and led us to suspect an esterase present in our enzyme preparation.

Polymyxin B and circulin were then incubated with 0.5 per cent *crystalline trypsin*<sup>7</sup> which showed proteolytic activity as tested by the Mett method (12). Inasmuch as an aqueous solution of crystalline trypsin is not destroyed on boiling, a boiled solution of the same concentration of crude trypsin served as the control. Crystalline trypsin failed to inactivate

<sup>5</sup> G. Brownlee, personal communication to Dr. B. E. Leach of this Laboratory.

<sup>6</sup> 1:250 trypsin obtained from Difco Laboratories, Inc., Detroit, Michigan.

<sup>7</sup> Obtained from Armour and Company, Chicago.

circulin or polymyxin B. Since crude trypsin was shown to contain lipase by the Kirsh method (13), the inactivation of these antibiotics is probably due to a fat-splitting enzyme.

In order to test this possibility a 1 per cent lipase preparation, free of proteolytic activity as tested by the Mett method (12), was incubated at 37° with circulin sulfate and polymyxin A, B, and D hydrochlorides at pH 7.8 to 8.0. Fig. 5 shows the rate of inactivation and is similar to that obtained by the action of crude trypsin. Circulin and polymyxin B were inactivated to the extent of 60 to 90 per cent in 5 to 10 days, while polymyxins A and D were unaffected. Even though circulin and polymyxin A are similar in chemical composition, as shown in Table IV, the above evidence demonstrates that they differ markedly in their resistance towards lipase.

It is interesting to note that on addition of alkali to a solution of circulin a white precipitate appears, which is insoluble in water. Simultaneously with the appearance of this biologically inactive precipitate, alkali is consumed, indicating the formation of an acid group. It is possible that this degradative cleavage is similar to the enzymatic inactivation.

Whether circulin is identical with the polymyxin E recently reported by Jones (4) remains to be studied. No polymyxin E or C was available at the time this investigation was in progress.

#### DISCUSSION

A chromatographic method with powdered cellulose, based on the paper strip technique, has been described. The method is readily applicable to simple mixtures of amino acids whose  $R_f$  values are sufficiently different from each other, and permits the isolation of the amino acid constituents in sufficient quantities for crystallization and detailed chemical identification.

Repeated chromatography and reversion to the sulfate from the picrate did not alter the chemical composition, specific rotation, or activity of circulin. On the basis of paper chromatography of the repurified material only one antibiotic was present. These criteria led us to believe that we were dealing essentially with a pure compound.

Based on our best analytical findings that circulin contains approximately 10 per cent leucine, 12 per cent threonine, 55 to 65 per cent  $\alpha, \gamma$ -diaminobutyric acid, and 10 per cent fatty acid, circulin would then contain 1 molecule each of D-leucine, L-threonine, and the fatty acid together with 5 molecules of L- $\alpha, \gamma$ -diaminobutyric acid. Assuming five free amino groups and a cyclic structure, the formula for the free base would be  $(C_{39}H_{74}O_9N_{12})_n$  or a minimum molecular weight of 854.

On the basis of nitrogen distribution, amino acid composition, and evidence that the  $\gamma$ -amino groups of  $\alpha, \gamma$ -diaminobutyric acid are the only

free amino groups in circulin, the amino acid distribution can be satisfactorily explained on the basis of a cyclic structure. In fact, evidence for the existence of a cyclic arrangement is also based upon the absence of a free carboxyl group, as is shown by a careful study of the titration curve.

Like polymyxin D (3) circulin has also been found to possess the  $\gamma$ -amino groups of  $\alpha,\gamma$ -diaminobutyric acid as the free basic amino groups. Since lipase affects only ester linkages, the fatty acid is most likely attached to the hydroxyl group of threonine.

It has been observed that one batch of crude circulin purified by carbon chromatography yielded two distinct and separable fractions whose amino acid composition, toxicity, and antibacterial spectra were identical with those of circulin previously described. However, the fatty acid fractions were not studied and in this respect the antibiotics may be different.

It is interesting that circulin produced by *Bacillus circulans* should prove to be a polypeptide similar to the polymyxins formed by *Bacillus polymyxa*. It is a conservative prediction that many more antibiotics of this nature will be found as metabolic products from other organisms than these.

#### SUMMARY

The chemistry of circulin, an antibiotic derived from a strain of *Bacillus circulans*, is described. The active principle was obtained in a purified form by repeated chromatography over carbon and regeneration from its salts. Circulin is a basic polypeptide and has the same amino acid composition as polymyxins A and E. However, it can be differentiated from polymyxin A by virtue of its inactivation by lipase. Polymyxin E was not available for these studies.

Circulin contains free amino groups, as exhibited by the Van Slyke and formalin reactions as well as by its reaction with 2,4-dinitrofluorobenzene.

Paper chromatography, microbiological, and isolation studies of acid-hydrolyzed circulin demonstrated that the antibiotic contains L-threonine, D-leucine, and L- $\alpha,\gamma$ -diaminobutyric acid. In addition to the amino acids an optically active isomer of pelargonic acid was found.

Paper chromatographic fractionation of the hydrolysate of 2,4-dinitrofluorophenyl-circulin demonstrated that the  $\gamma$ -amino groups of  $\alpha,\gamma$ -diaminobutyric acid account for all of the free amino groups in circulin. Furthermore, all of the  $\gamma$ -amino groups are free, as is testified by the fact that approximately one-half of the amino nitrogen of circulin is uncombined.

A technique was developed for separating the amino acids on a column of powdered cellulose. The behavior on the column resembles that of the paper chromatogram. This technique provides a preparative method for isolation of amino acids and should be extremely useful when either the

paper strip chromatogram or optical rotation, or both are employed as the indicator.

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# PAPER CHROMATOGRAPHY OF VITAMIN B<sub>12</sub> AND RELATED BACTERIAL GROWTH FACTORS\*

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In a preliminary note (1), the present authors reported that *Lactobacillus leichmannii* 313 is capable of using six alternative factors for growth in vitamin B<sub>12</sub>-deficient media. Two of these were found in vitamin B<sub>12</sub> concentrates and presumably represent two forms of the vitamin. Hoffmann *et al.* (2) previously noted that crystalline antipernicious anemia factor (APA) (vitamin B<sub>12</sub>) supports the growth of *L. leichmannii* 313. Of the other four factors, one was provisionally identified as thymidine, a substance which was reported by Snell *et al.* (3) as supporting the growth of this organism. The three remaining growth factors are of unknown composition.

In the present report, a paper chromatographic procedure for separating the alternative growth factors is described as well as the method of recognizing the positions of the several factors on a chromatogram by use of *L. leichmannii* 313 as a microbiological indicator. Extension of the method to the examination of other natural materials has revealed the existence of at least one other substitute growth factor replacing vitamin B<sub>12</sub> in the nutrition of the test organism.

That the naturally occurring alternative growth factors which replace vitamin B<sub>12</sub> in the nutrition of *L. leichmannii* 313 are desoxyribosides would appear probable from evidence presented in this paper. Kitay *et al.* (4) have shown that desoxyribosides can replace thymidine in the nutrition of several lactic acid bacteria.

The paper chromatographic technique has aided in the interpretation of assay values for apparent vitamin B<sub>12</sub> activity as measured by the usual tube assay.

## EXPERIMENTAL

*Paper Chromatography and Development of Bioautographs*—The general method involving the use of paper chromatography in the analysis of growth factors which occur in nature in more than one chemically defined form has been described by Winsten and Eigen (5). Briefly, what has

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been termed the bioautographic technique consists of subjecting a droplet of a solution containing the substitute growth factors to paper chromatography. The resultant paper chromatogram is then laid on the surface of nutrient agar seeded with an appropriate bacterial culture. The nutrient agar contains all other factors necessary for the growth of the test organism, except any one of the complex of substances being studied. After removal of the paper strips and incubating, zones of microbial growth form at different positions along the locus of the strip chromatogram. The positions of the various substitute growth factors depend on their *R<sub>F</sub>* values for the solvent system employed in developing a chromatogram and serve to characterize and identify the different factors present in the

TABLE I  
*Vitamin B<sub>12</sub> Basal Medium (Double Strength)*

DL-Tryptophan	800 mg.	Biotin	0.8 γ
L-Cystine	400 "	Pyridoxine hydrochloride	2400 "
DL-Alanine	400 "	Calcium pantothenate	800 "
L-Asparagine	200 "	Folic acid	1000 "
Glutamine	40 "	<i>p</i> -Aminobenzoic acid	20 "
Adenine	20 "	Casein hydrolysate*	100 ml.
Guanine	20 "	" digest†	25 "
Uracil	20 "	Dextrose	40 gm.
Xanthine	20 "	Sodium acetate	40 "
Pyridoxal hydrochloride	500 γ	K <sub>2</sub> HPO <sub>4</sub>	5.0 "
Thiamine "	400 "	Tween 80	2.0 "
Riboflavin	400 "	Salts B‡	10 ml.
Niacin	1200 "	Adjust to pH 6.8 and add distilled water to make 1 liter	

\* Acid-hydrolyzed casein obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

† Enzymatic digest of casein obtained from Nutritional Biochemicals.

‡ MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 gm.; NaCl, 0.5 gm.; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 gm.; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.5 gm.; dissolved in 250 ml. of distilled water.

original sample. The incubated plate which reveals the developed chromatogram by this direct microbiological procedure has been termed a bioautograph.

In the present study on vitamin B<sub>12</sub> and substitute growth factors, 10 to 30 μl. samples of the solutions being examined (pH 5.0) were spotted on Whatman No. 1 paper strips. The chromatograms were developed overnight at room temperature with wet *n*-butanol in the apparatus described by Winsten (6). The strips were allowed to dry in air for 1 hour at 30–35°. They were then laid on agar plates seeded with *L. leichmannii* 313. The composition of the double strength basal medium is described in Table I. The medium was diluted 2-fold, and 1.5 per cent agar was

added. The medium is fundamentally similar to that suggested by Hoffmann *et al.* (2).

The organism *L. Leichmannii* 313 was maintained on Difco tryptose agar slabs prepared weekly. The daily transplants were made in a broth containing 2 per cent tryptose and 2 per cent litmus milk. The cultures were incubated at 37°.

For use on the agar plates, a 24 hour culture of the organism in the tryptose-milk broth was transplanted to 10 ml. of single strength basal medium to which 2  $\mu$ gm. of vitamin B<sub>12</sub> were added. After 24 hours, the culture was centrifuged for 15 minutes at 2500 R.P.M. and was resuspended in 10 ml. of physiological saline. The culture was again centrifuged and suspended in 10 ml. of saline. The resultant suspension was then added to 300 ml. of melted basal medium maintained at a temperature of 42–50°. The seeded agar was poured on rectangular plates 9.5 × 16 inches.

After laying the dried paper strip chromatograms on the hardened agar and allowing the moist agar to leach the strips for 5 minutes, they were removed and the plate was incubated overnight at 37°. The resulting zones of bacterial growth were always quite light and, in order to facilitate observation, it was necessary to hold the plate at an angle with a source of light to one side and behind it. When so observed, the zones were always sharply defined, well formed ellipses. The edges were outlined in the agar with a sharp instrument. Contact photographic prints were then prepared with Kodagraph contact standard paper. The zones were finally marked in ink on the contact paper, making a permanent record.

Samples of the various materials analyzed contained 0.1 to 10.0  $\gamma$  per ml. calculated as vitamin B<sub>12</sub>, determined by a titrimetric tube assay procedure with *L. leichmannii* 313.

On occasion, samples were first subjected to the proteolytic action of protease 15, an enzyme preparation manufactured by Rohm and Haas. Usually 5 ml. of a suspension (or solution) of material calculated to give a final solution containing the above range of apparent vitamin B<sub>12</sub> activity were treated with 10 mg. of protease at pH 7.0 for 18 hours at 37° in the presence of toluene.

Grateful acknowledgment is made for the various materials analyzed, among which may be mentioned a vitamin B<sub>12</sub> concentrate and a preparation of crystalline vitamin B<sub>12</sub> supplied by Dr. W. L. Sampson and Dr. W. H. Ott of Merck and Company; crystalline APA (in solution), a fermentation animal protein factor (APF) preparation, and a thymidine sample supplied by Dr. T. H. Jukes and Dr. A. L. Franklin of the Lederle Laboratories; an acid precipitate of the cow manure factor from Dr. H. R. Bird; and a sample of hypoxanthine desoxyriboside supplied by Dr. E. E. Snell. In addition, several commercial parenteral liver preparations for



use in the treatment of pernicious anemia were examined. Other materials tested included condensed fish solubles, a commercial stomach concentrate intended for use in pernicious anemia, corn steep liquor, a commercial trypsin, Wilson's liver powder 1:20, and an enzyme digest of sperm desoxyribonucleic acid.

*Results of Qualitative Bioautographic Analysis for Vitamin B<sub>12</sub> and Alternative Growth Factor*—Fig. 1 shows a schematic diagram of a typical set of

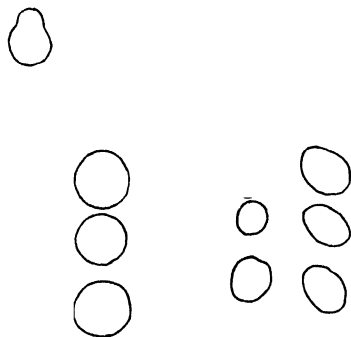


FIG. 1. Bioautographs of preparations containing vitamin B<sub>12</sub> together with substitute growth factors. Test organism, *L. leichmannii* 313. Chromatograms developed for 18 hours at room temperature with wet *n*-butanol; 10 to 20  $\mu$ l. samples were chromatographed. Strip 1, crystalline vitamin B<sub>12</sub> preparation, 3.8  $\gamma$  per ml.; Strip 2, parenteral liver Preparation 1, 0.67  $\gamma$  per ml. of apparent vitamin B<sub>12</sub> content; Strip 3, corn steep liquor, about 0.25  $\gamma$  per gm. of apparent vitamin B<sub>12</sub>, diluted 1:2; Strip 4, corn steep liquor, diluted 1:5; Strip 5, vitamin B<sub>12</sub> Concentrate 2, 1.5  $\gamma$  per ml. of apparent vitamin B<sub>12</sub>.

bioautographs obtained as described above. From an examination of Strip 2, which represents the bioautograph of parenteral liver Preparation 1, it will be noted that the slowest moving zone (at the top of the strip) has a double character, suggesting two substances. Analysis of crystalline vitamin B<sub>12</sub> preparations and vitamin B<sub>12</sub> concentrates indicates that the doublet zone of growth may be due to two forms of vitamin B<sub>12</sub>. In this connection it is of interest to recall the two forms of APA factor reported by Smith (7).

Further examination of Strip 2 reveals the presence in this preparation of three other substances which move more rapidly on the chromatogram. The nature of these factors will be discussed below.

It was found that the  $R_F$  value for a given growth factor present in several preparations was independent of the type of preparation examined within experimental error. Thus on a given day the  $R_F$  value of the most rapid factor was 0.55 for parenteral liver Preparation 1, 0.54 for parenteral liver Preparation 2, and 0.54 for parenteral liver Preparation 3. However, from day to day the  $R_F$  value varied anywhere from 0.52 to 0.67. As a consequence, in identifying new factors on any given day a well studied material (the parenteral liver Preparation 1) was also analyzed. It was further found that, if the most rapidly moving substance was assigned a value of 1.0 for its rate of movement, the rates of movement of the other substances relative to it were reasonably constant. This was true so long as the daily temperature variations were minor. Toward the end of the present study, with the onset of the higher summer laboratory temperatures, the relative rates changed somewhat in a non-uniform manner. This may be a consequence of the fact that the percentage change of  $R_F$  value with temperature was not the same for all the alternative growth factors.

In Table II are summarized the relative rates of movement of the various alternative growth factors, the most rapidly moving substance found in the thymidine preparation being assigned a rate of 1.0.

Included in the vitamin B<sub>12</sub> column of Table II are all factors which were so slow moving, when wet *n*-butanol was the developing solvent, that accurate  $R_F$  values could not be obtained. On occasion, only a single zone of growth was observed on a bioautograph at the site of application of the test sample.

It is evident that vitamin B<sub>12</sub> may be more complex in character than is indicated by the terms double and single zones of growth. Clearly a more efficient solvent than wet *n*-butanol is needed to resolve the substances that cause the characteristic double zone of growth associated with so called crystalline vitamin B<sub>12</sub> preparations. Experimental results obtained with such a solvent will be described in a later publication.

It is of interest that all preparations so far tested, which were intended for the treatment of pernicious anemia, contained a growth factor or factors which moved slowly or not at all when *n*-butanol was used as a solvent. Alternative growth factors of greater solubility in wet *n*-butanol relative to water were found in many of the cruder pernicious anemia preparations as well as in preparations of fermentation APF and condensed fish solubles.

In an investigation of corn steep liquor, one factor was found which did not fit in with those indicated in Table II. In Fig. 1 are shown bioautographs for two dilutions of corn steep liquor (see Strips 3 and 4).

It is evident, on comparing these with the bioautograph for parenteral liver Preparation 1, that at least one of the two slowest moving growth factors in corn steep liquor must represent a fifth alternative growth factor in addition to the four of Table II. The average relative rates of the growth in corn steep liquor were 0.51, 0.63, 0.76, and 1.0. Inspection of

TABLE II  
*Relative Rates of Movement of Substitute Growth Factors for L. Leichmannii 313\**

Preparation	Vitamin B <sub>12</sub> † (type of zone of growth)	Growth factors present			
		Substitute factors			
		1	2	3	4
		Relative rates in terms of Factor 4			
Parenteral liver Preparation 1	Double		0.54	0.78	1.0
“ “ “ 2	“		0.51	0.77	1.0
“ “ “ 3	“	0.32	0.54	0.83	1.0
Trypsin (1%, crude)			0.51		1.0
Vitamin B <sub>12</sub> Concentrate 1	Double				
“ “ “ 2	“		0.56	0.77	1.0
“ “ crystalline	“				
Cow manure factor	Single (no movement)				
Fermentation APF preparation	Double		0.56	0.76	1.0
Wilson liver preparation, 1:20 (10%)	“			0.80	1.0
Stomach concentrate (enzyme-treated)	Single (no movement)		0.52	0.78	1.0
Condensed fish solubles	Single (no movement)		0.50		1.0
Crystalline APA factor	Double				
Thymidine				0.75‡	1.0
Average.....		0.32	0.53	0.78	1.0

\* Chromatography on Whatman No. 1 paper overnight with wet *n*-butanol as the solvent.

† The two forms of vitamin B<sub>12</sub> moved too slowly, preventing an accurate measurement of their relative rates. On occasion bioautographs exhibit a single zone in the region normally occupied by vitamin B<sub>12</sub> in place of the double most often observed.

‡ Very small zone.

the average values in Table II suggests that the factor in corn steep liquor with a relative rate of 0.63 represents the new substance. Thus, in addition to at least two forms of vitamin B<sub>12</sub>, five other substitute growth factors are present in various natural materials.

*Evidence of Desoxyribose Nature of Alternative Growth Factors*—That the

substitute growth factors dealt with in Table II represent desoxyribosides appeared probable, as mentioned earlier in this paper. To throw light on this question, we attempted to identify one of the factors with thymidine, the only desoxyriboside available to us at the start of this investigation. From Table II it is apparent that the thymidine preparation contained two factors. While it seemed probable that the most rapid factor (assigned relative rate, 1.0) which produced the larger zone of growth was thymidine, proof of this fact was lacking.

Since only a small amount of thymidine was available, the method of Hotchkiss (8) could not be applied. A second indirect method was available, however. By the use of *Leuconostoc citrovorum* 8081 in a bioautographic method, an organism which utilizes thymidine in place of an uncharacterized factor, as described by Sauberlich and Baumann (9), it was found that the thymidine preparation caused a zone of growth at a position identical, within experimental error, with that of the faster, larger zone obtained when *L. leichmannii* 313 was the test organism.

The slower factor in the thymidine preparation (see Table II) was inactive for *L. citrovorum* 8081. These findings therefore serve to identify the factor assigned a relative rate of 1.0 as thymidine.

Subsequent bioautographic testing of hypoxanthine desoxyriboside indicated that the preparation, kindly made available by Dr. E. E. Snell, was free of any other factors and that this desoxyriboside moved with a relative rate, indicating its possible identity with Substitute Factor 2 in Table II.

Additional evidence suggesting the desoxyriboside nature of the more rapidly moving growth factors other than vitamin B<sub>12</sub> was afforded by bioautographic analysis of an enzyme digest of desoxyribonucleic acid. The digestion was carried out substantially in the manner described by Chargaff *et al.* (10) for the production of desoxyribonucleosides.

A sample of sperm desoxyribonucleic acid (DNA), obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, was dissolved with the aid of a little alkali to give a 4 per cent solution. The pH was adjusted to 6.7. Bioautographic analysis of this solution of DNA revealed the presence of a vitamin B<sub>12</sub>-like factor (*i.e.*, it did not "move" on a paper chromatogram when *n*-butanol was the solvent).

To a 1 ml. sample of the DNA solution, 1 ml. of veronal buffer of pH 6 to 7, containing 2 per cent MgSO<sub>4</sub>·3H<sub>2</sub>O, and 1 ml. of an enzyme solution were added. The enzyme solution contained 300  $\gamma$  per ml. of crystalline desoxyribonuclease (Worthington Biochemical Laboratory) and 50 mg. per ml. of mylase P (Wallerstein Laboratories). After incubation at about 30° overnight under toluene, bioautographic analysis showed the presence of four substitute growth factors in addition to the vitamin B<sub>12</sub>-like factor

originally present before enzyme digestion. A control analysis of the enzyme solution indicated the substantial absence of such factors.

The relative rates of movement of the factors were 0.57, 0.69, 0.78, and 1.00. The relative rates of the substitute growth factors in parenteral liver Preparation 1, subjected to bioautographic analysis at the same time as the enzyme digest of DNA, were 0.60, 0.79, and 1.00. The first figure (0.60) for liver Preparation 1 is higher than that indicated in Table II for the same liver preparation. As was pointed out earlier, this is probably a consequence of a temperature change in the laboratory. The data of Table II were obtained at about 25°. The temperature in the present experiment was much higher (about 31°). If we take account of this change in relative rates, the factors found in the DNA digest are possibly hypoxanthine desoxyriboside (relative rate 0.57) and thymidine (relative rate 1.0).

The other two factors (relative rates 0.69 and 0.78) may represent other desoxyribosides in the enzyme digest of DNA. For brevity, a diagram of the bioautograph of the DNA digest has not been given. The main factors present in the digest (*i.e.*, the ones causing the largest zones of growth) were those with the relative rates 0.57 and 1.0. This finding may have a bearing on the relative rates of liberation of the desoxyribosides by enzyme digestion, and on their relative activity for *L. leichmannii* 313.

The four factors found in corn steep liquor may represent the same desoxyribosides found in the DNA enzyme digest. Their occurrence in corn steep liquor may be a consequence of bacterial fermentation, since the corn steep liquor examined was highly fermented.

The only factor which so far has not been implied to be a desoxyriboside is that one with a relative rate of 0.32 (see Table II). That it is such a substance seems probable.

*Quantitative Analysis of Vitamin B<sub>12</sub> and Substitute Growth Factors in Parenteral Liver Preparation*—It has been shown above that it is possible to separate vitamin B<sub>12</sub> and various alternative growth factors by the use of paper chromatography. Assuming that all the growth factors which barely move away from the site of application of a test sample on a chromatogram (when wet *n*-butanol is used as a developing solvent) represent forms of vitamin B<sub>12</sub>, it is possible to assay such factors in various concentrates in a simple fashion. Such an assay was carried out on parenteral liver Preparation 1 (see Fig. 1, Strip 2). In this assay, several 8.6  $\mu$ l. samples of the parenteral liver preparation were subjected to chromatographic separation on paper strips in the usual way. After being dried in air, each strip was cut in two at a point just below the estimated position of the vitamin B<sub>12</sub> entities (about 1.5 inches beneath the site of application of the test sample). The upper sections of the strip chromatograms therefore contained the slow moving vitamin B<sub>12</sub>. The lower sections contained the faster moving alternative growth factors.

The top and bottom sections were each eluted with a known volume of water and aliquots were taken for assay of total apparent vitamin B<sub>12</sub> activity. In carrying out the assay, a 72 hour titrimetric tube assay was employed with the basal medium given in Table I.

In addition to the above, as controls, unchromatographed samples were also assayed. As a further check, instead of cutting some of the strip chromatograms after development, the entire strips were eluted and assayed. Finally, in order to demonstrate that there is no loss in vitamin B<sub>12</sub> during chromatography, several samples of vitamin B<sub>12</sub> Concentrate 1 were also chromatographed; the chromatograms were cut and the top and bottom sections were assayed for vitamin B<sub>12</sub>. Every assay was carried out in

TABLE III

*Quantitative Assay of Fractions of Parenteral Liver Preparation 1 after Separation on Paper Chromatograms*

Material analyzed		Apparent activity calculated as crystalline vitamin B <sub>12</sub> , $\gamma$ per ml.				Average
		Determination 1	Determination 2	Determination 3	Determination 4	
Parenteral liver Preparation 1*	Not chromatographed	0.67	0.70	0.67	0.64	0.67
	Top of chromatogram	0.73	0.80	0.80	0.66	0.75
	Bottom of chromatogram	0.19	0.17	0.17	0.19	0.18
	Entire chromatogram eluted	0.76	0.73	0.73	0.73	0.74
Vitamin B <sub>12</sub> Concentrate 1	Not chromatographed	2.08	2.00	1.93	1.93	1.99
	Top of chromatogram	2.00	2.08	2.08		2.03
	Bottom of chromatogram	None	None	None	None	

\* In carrying out determinations on the cut sections of the chromatograms, two top sections and two bottom sections were eluted together; in all eight chromatograms were sectioned and combined in this way for the four determinations.

quadruplicate with crystalline vitamin B<sub>12</sub> as the standard. The results appear in Table III.

It will be noted that after chromatography there was somewhat more apparent vitamin B<sub>12</sub> activity per ml. at the top of the chromatogram compared to the value obtained without chromatography. The bottom section of the chromatogram, containing as it did the three fast moving alternative growth factors, contributed an additional amount of apparent vitamin B<sub>12</sub> activity, which was only about 25 per cent of the activity at the top of the chromatogram.

It would appear, in the present instance at least, that, in a total tube assay of an unchromatographed sample, the value obtained (0.67  $\gamma$  per ml.) was not the sum of the activities contributed by the top and bottom sets of factors measured separately. This finding raises a question concerning

the joint growth-promoting effects of vitamin B<sub>12</sub> and thymidine. While several authors have noted that these substances can replace one another in supporting the growth of certain lactic acid bacteria, there is little detailed information on the quantitative aspects of their combined action. Lack of sufficient pure thymidine has prevented us from obtaining pertinent data on this problem.

From Table III, it is also plain that little or no vitamin B<sub>12</sub> is lost during the development of the paper chromatograms, since all of the vitamin applied as Concentrate 1 was recovered by eluting the top sections of the chromatograms.

In Fig. 1, consideration of the size of the zones of growth in the bioautograph for the parenteral liver Preparation 1 (see Strip 2) would lead one to believe that most of the activity would have been found in the bottom sections of the chromatograms of Table III. The small area of the double zone of growth caused by the very slow moving vitamin B<sub>12</sub> is probably due to poor diffusion in the agar because of its high molecular weight.

*Note*—Stokstad *et al.* (11) have presented evidence that, when vitamin B<sub>12</sub> is autoclaved with a basal medium, there is some destruction of the vitamin. The amount of destruction varies with the preparation being analyzed. These authors pointed out that inclusion of thioglycolic acid (TGA) in the medium protected vitamin B<sub>12</sub> from destruction by autoclaving. In the light of these observations, it was of interest to repeat the experiment, the results of which were described in Table III. The vitamin B<sub>12</sub> content of an unchromatographed sample of parenteral liver Preparation 1, determined in the presence of TGA during autoclaving, was 0.60  $\gamma$  per ml., about the same as is indicated in Table III. (Other parenteral preparations exhibited anywhere from 50 to 100 per cent more vitamin B<sub>12</sub> activity when TGA was present during autoclaving, confirming the findings of Stokstad *et al.*) Quadruplicate assay of the top of a chromatogram, in the presence of TGA, yielded a value of 0.80  $\gamma$  per ml., again in agreement with the value of Table III. Assay of the bottom of a chromatogram in the presence of TGA yielded 0.02  $\gamma$  per ml., only about 10 per cent of the figure noted in Table III. Thus the apparent vitamin B<sub>12</sub> activity of faster moving alternative growth factors was much less when measured in the presence of TGA.

#### DISCUSSION

The application of the bioautographic method to the study of vitamin B<sub>12</sub> and substitute growth factors for *L. leichmannii* 313 has revealed marked similarities among such diverse materials as parenteral liver preparations intended for the treatment of pernicious anemia, a fermentation APF preparation, and condensed fish solubles. Fish solubles have been used by many authors as sources of APF for chicks. While it has

been implied that the APF activity of fish solubles may be due to the vitamin B<sub>12</sub> content of the preparation, no evidence other than the growth-promoting effect for chicks and perhaps for lactic acid bacteria has been presented. In the present paper, the finding in condensed fish solubles of one factor that does not "move" on a paper chromatogram under the conditions described, which is thus similar to authentic vitamin B<sub>12</sub>, has been demonstrated. In addition there are present two other more rapidly moving growth factors, found on occasion in a wide variety of other sources of vitamin B<sub>12</sub>.

As mentioned earlier, the question has yet to be answered as to whether the more mobile growth factors for *L. leichmannii* 313, which are probably desoxyribosides, have APF or antianemia activity. The more mobile factors by themselves display apparent vitamin B<sub>12</sub> activity in preparations, such as corn steep liquor, which lack the vitamin itself as evidenced by the absence of any "immobile" growth factor. This finding suggests that all studies on the apparent vitamin B<sub>12</sub> content of natural materials determined in the usual form of tube assay should be checked by a bioautographic study to ascertain the types of growth factors present.

It has recently been reported by Nichol *et al.* (12) that one can obtain liver preparations which are active in the treatment of pernicious anemia but possess no animal protein factor activity for the chick. It would be of great interest to determine what growth factors, if any, for *L. leichmannii* 313 are present in such preparations.

#### SUMMARY

1. A paper chromatographic study of vitamin B<sub>12</sub> and related growth factors has been carried out. In addition to vitamin B<sub>12</sub>, five other substitute growth factors for *Lactobacillus leichmannii* 313 have been recognized. Evidence of the desoxyriboside nature of the substitute growth factors has been presented.

2. A quantitative analysis for vitamin B<sub>12</sub> which comprises the joint use of paper chromatography and a tube assay of the separated factors has been described.

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# 1-5-VINYL-2-THIOOXAZOLIDONE, AN ANTITHYROID COMPOUND FROM YELLOW TURNIP AND FROM BRASSICA SEEDS\*

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Extensive studies have been made on the effects of various articles of the diet on the induction of goiter in animals. Certain plants, such as cabbage (1), turnip (2), rape seed (3), soy bean (4), and peanut (4), have been reported to cause thyroid enlargement. Sometimes added iodine seemed to prevent the development of these goiters, but in other instances it did not. Although it has often been suggested that the effect of these foods may be attributable to a goitrogenic compound contained in them, attempts to isolate an active principle have heretofore been unsuccessful.

Only two antithyroid compounds have been authentically reported to occur in plants. Klein and Farkass (5) have detected thiourea by microchemical methods in *Laburnum anagyroides*. In 1938 Hopkins (6) obtained 5,5-dimethyl-2-thiooxazolidone from seeds of the crucifer *Conringia orientalis*; this compound was approximately one-fifth as active as thiouracil in the rat (7). On the other hand, the alleged existence (8) of benzylthiourea in seeds of *Carica papaya* is questionable. For the isolation, the papaya seeds, which contain an unidentified mustard oil (9, 10), were extracted with an ammoniacal medium, Prollius' fluid (11); hence, the benzylthiourea found may have been an artifact.

In a recent investigation (12) of the antithyroid effects of foods in normal subjects, several foods were found to inhibit the uptake of radioactive iodine in a manner similar to that of antithyroid compounds. The most effective of these was rutabaga (yellow turnip or Swedish turnip), which was subsequently utilized for isolation of the active principle.

**Isolation**—The antithyroid activities of preparations of the rutabaga factor were followed during early stages of purification by a simplified rat assay with  $I^{131}$ . Partially concentrated fractions, in contrast to the crude extract, gave a pink or purple color with Grote's reagent (13) similar to that produced by many thio compounds. Since most known sulfur-con-

\* Aided in part by grants from the United States Public Health Service and from the American Cyanamid Company.

† Member of the Society of Fellows, Harvard University.

taining goitrogens are thioamides and exhibit ultraviolet absorption, the rutabaga concentrates were examined and found to possess a strong absorption maximum, at 240 m $\mu$ , of intensity parallel to antithyroid activity. Consequently, the final purification of the active principle was controlled by spectrophotometric assay.

As previously reported (12), the antithyroid factor was extracted from finely ground, raw rutabaga root by steeping in cold water, was stable

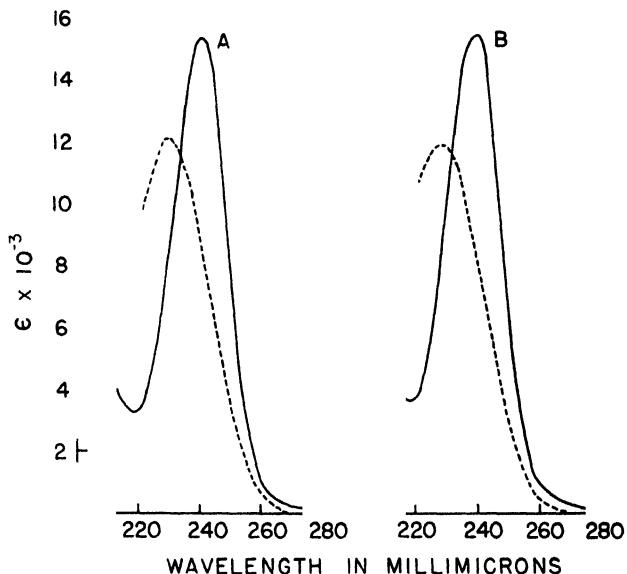
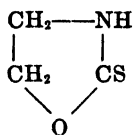


Fig. 1. Ultraviolet absorption spectra of the turnip factor (A) and of 2-thiooxazolidone (B) in distilled water (solid lines) and in 0.1 N sodium hydroxide (dotted lines). The spectrum of 5,5-dimethyl-2-thiooxazolidone was nearly identical with that of B.

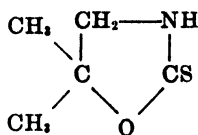
during evaporation of the filtrate, and could be extracted from the aqueous concentrate by organic solvents, of which ether proved to be the most selective. Furthermore, the substance was freely soluble in water or dry ether, was distributed between water and ether in approximately unit ratio, and was extracted from ether by strong alkali, properties which permitted its separation as a slightly colored oil of 60 per cent purity. After additional processing by high vacuum distillation and chromatography on alumina, a fraction was induced to solidify. Once seed crystals had been obtained, the concentrates prepared by solvent partition could be completely purified by direct crystallization from ether.

**Determination of Structure**—The antithyroid factor of rutabaga was isolated as colorless, optically active crystals, m.p. 50°,  $[\alpha]_D^{25} = -70.5^\circ$

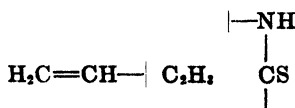
(methanol). Analyses and a molecular weight determination furnished the molecular formula,  $C_5H_7ONS$ . No compound of this formula and with similar properties had hitherto been described. The compound was a weak monoacid,  $pK_a$  10.5, had no basic properties, and was stable in hot alkali but not in acid. The ultraviolet absorption spectrum (Fig. 1) in aqueous solution exhibited a single intense maximum at  $240\text{ m}\mu$ ,  $\log \epsilon$  4.18, shifted by alkali to  $232\text{ m}\mu$ ,  $\log \epsilon$  4.08. Nearly identical acidities and spectra were found to be possessed by the model compounds, 2-thioxazolidone (I) and 5,5-dimethyl-2-thioxazolidone<sup>1</sup> (II). Therefore, the physical properties of the rutabaga factor, like its antithyroid activity, suggested the presence of a thioncarbamate group,  $-\text{NH}-\text{CS}-\text{O}-$ . Final evidence in this direction was afforded by the infra-red absorption spectrum (Fig. 2) of the unknown. The system of bands (stars) at  $2.92$ ,  $3.17$ ,  $6.63$  (inflected at  $6.5$ ), and  $8.57\text{ }\mu$ , which is characteristic of the thioncarbamate group present in thioxazolidones, conclusively established the function of the hetero atoms.



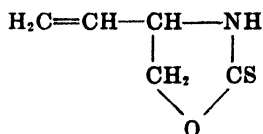
(I)



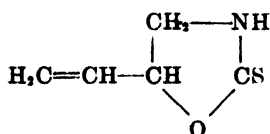
(II)



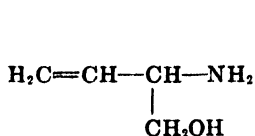
(III)



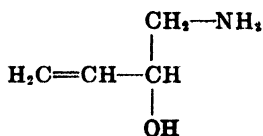
(IV)



(V)



(VI)



(VII)

Another significant feature in the infra-red spectrum was the pair of bands (arrows; Fig. 2) at  $10.17$  and  $10.88\text{ }\mu$ , which indicated a vinyl group (14, 15). The partial structure III could therefore be written, wherein a ring was required in order to permit optical activity. Since the possibility of a 4-membered ring could be dismissed, the substance was either 4- or 5-vinyl-2-thioxazolidone (IV or V). The observed absence of *C*-methyl groups further strengthened this deduction.

<sup>1</sup> The previously published spectrum of this substance appears to be inaccurate.

A decision between the two remaining structural possibilities was made as follows. On acid hydrolysis, compounds IV and V would yield the amino alcohols VI and VII respectively, which still possess an asymmetric carbon atom. Allylic amines (16, 17) and 1,2-amino alcohols (18, 19) are stable in strong acid; hence, the amino alcohol VI would be expected to retain optical activity, which it could not lose without concomitant loss of ammonia. On the contrary, the amino alcohol VII, in accord with the known sensitivity of allylic alcohols to acid (20-22), should suffer attack and racemization as it is formed. Experimentally, upon cleavage of the rutabaga compound with boiling 4 N hydrochloric acid, the optical activity disappeared and ammonia could not be detected as a product. Therefore, the antithyroid factor of rutabaga was proved to be *l*-5-vinyl 2-thiooxazolidone (V). This structure was subsequently confirmed by synthesis.<sup>2</sup>

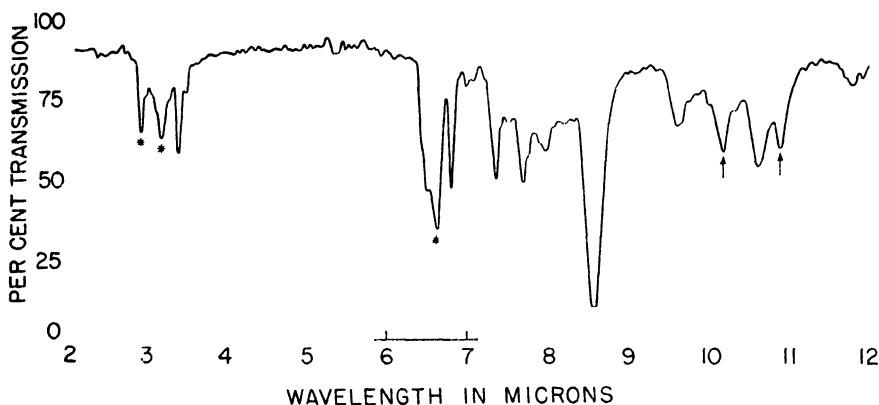


FIG. 2. Infra-red absorption spectrum of the turnip factor (chloroform solution). Bands characteristic of the 2-thiooxazolidone and vinyl groups are indicated by stars and arrows respectively.

The spectrophotometrically determined distribution of the compound in the seeds of some members of the Cruciferae is shown in Table I. It was not detected in any plant except those of the genus *Brassica*, and within that genus it was absent from the seeds of mustard and cauliflower. It was isolated from the edible root of rutabaga and white turnip in amounts which varied from 0.12 to 1.0 gm. per kilo, but it could not be detected in the edible portions of other *Brassica* vegetables, including cabbage, Chinese cabbage, kale, cauliflower, broccoli, mustard-greens, or horseradish root. The possible biogenetic relation of the antithyroid factor to the unsaturated 5-carbon mustard oil of rape seed (23, 24) merits further exploration.

The compound appeared to be present in the plant as a water-soluble,

<sup>2</sup> Ettlinger, M. G., to be published.

ether-insoluble precursor, from which the thiooxazolidone was formed apparently by enzymatic action. Boiling the root or seed in water inactivated the enzyme, and no biological activity could be detected; nor could any thiooxazolidone be isolated. The compound could be liberated from heated material by treatment with fresh plant tissue or with protein fractions thereof.

TABLE I  
Thiooxazolidone Content of Seeds of Plants of Mustard Family

Genus	Plant	No. of varieties tested	Content	
			Range	Mean
			<i>gm. per kg.</i>	<i>gm. per kg.</i>
<i>Brassica</i>	Rutabaga*	7	0.8-8.6	2.5
	Turnip*	9	0.3-2.5	1.0
	Cabbage*	9	0.2-4.7	1.5
	Kale*	3	0.9-6.3	4.4
	Rape* ✓	3 -	1.8-2.1	1.9
	Chinese cabbage	1		0.7
	Brussels sprouts	2	0.5-0.8	0.7
	Broccoli	1		1.6
	Kohlrabi	2	0.7-1.4	1.1
	Cauliflower	2		0
	Mustard ✓	3		0
<i>Raphanus</i>	Radish	12		0
<i>Lobularia</i>	Sweet alyssum	5		0
<i>Matthiola</i>	Stock			0
<i>Iberis</i>	Candytuft	2		0
<i>Nasturtium</i>	Cress	1		0
<i>Lepidium</i>	"	1		0
<i>Arabis</i>	Rock cress	1		0
<i>Cheiranthus</i>	Wallflower	2		0
<i>Lunaria</i>	Honesty	1		0

\* Identified by isolation.

Tested in normal human subjects by use of radioactive iodine (25), the compound was found to have an antithyroid activity equal to that of thiouracil. Single doses of 50 mg. in two subjects exerted a marked but not complete inhibition of iodine uptake by the thyroid gland; doses of 100 mg. in four subjects were completely inhibitory for 4 hours or longer, and doses of 200 mg. in two subjects were completely inhibitory for 24 hours.

The usual rat method of assay involving the administration of the compound for 10 days admixed with the food (7) and a method in rats with

the use of  $I^{131}$  showed it to be one-fifth as active as thiouracil and similar in activity to 2-thiooxazolidone and 5,5-dimethyl-2-thiooxazolidone.

#### EXPERIMENTAL

*Assay Method*—Several tests on the starting material and on partially purified extracts were made in man by the methods previously described (25). For most assays a test in immature rats with radioiodine was employed. The method was similar to that used by McGinty *et al.* (26) for the assay of antithyroid compounds. The ground starting material was administered by stomach tube, but the more purified fractions were given by subcutaneous injection. 1 hour later a tracer dose of carrier-free  $I^{131}$  was injected subcutaneously, and 3 hours thereafter the thyroid gland was excised and placed on a piece of dry absorbent paper not quite covering a standard 1 in. copper disk. The gland was secured on the disk by Scotch tape and pressed flat with a heavy weight. The  $I^{131}$  content was determined by counting  $\beta$  radiation with a Geiger-Müller counter. Thiourea and thiouracil were used as standards.

*Extraction and Purification*—5 kilos of turnip roots or 500 gm. of the seed were ground in a mechanical grinder or in a Waring blender. The ground seed was suspended in 5 liters of water, but the root required no additional water. The mash was allowed to stand at room temperature with occasional stirring for several hours or overnight. The suspension was then coagulated by heating to above  $60^{\circ}$ , pressed through cloth, and filtered. The residue was reextracted with one-half of the original volume of water, and the combined filtrates were evaporated to a thin syrup, either by vacuum distillation or by heating in shallow pans on a steam bath in a stream of air. The active principle was recovered from the aqueous concentrate by five extractions with equal volumes of peroxide-free ether. The combined ether extracts were reduced to a convenient volume of about 2 liters, and shaken six times with 10 cc. portions of saturated sodium bicarbonate solution. A negligible amount of the active principle was lost in this step, and virtually the whole of the desired substance was then removed from the ether by six extractions with 10 cc. portions of 2 N sodium hydroxide. The combined sodium hydroxide extracts were neutralized to a pH of 8 to 8.5 with carbon dioxide and extracted five times with equal volumes of ether. After removal of the ether by evaporation, the yellow oily residue was thoroughly shaken with 50 cc. of water. The turbid aqueous phase was removed from an insoluble deeply colored tar, treated with charcoal, and filtered. The clear and nearly colorless filtrate was evaporated to an oily residue which was thoroughly dried *in vacuo*. The oil was dissolved in 10 to 20 cc. of dry, peroxide-free ether, leaving a small amount of insoluble solid; the solution was treated with charcoal and

filtered. The ether solution was evaporated to about 5 cc., chilled in the freezing chamber of a refrigerator, and seeded. The crystals were collected after several hours at  $-10^{\circ}$  and recrystallized one or more times. The yield of crystalline material averaged about 50 per cent of that contained in the seed or root, measured spectrophotometrically.

Crystals were initially obtained from material which had been further purified by vacuum distillation and by chromatographic adsorption. An oily extract, estimated to contain 600 mg. of the substance, was heated under the vacuum of a mechanical pump in a small spherical flask in a water bath, and the distillate was collected on a test-tube projecting into the center of the flask containing an alcohol-dry ice mixture. As the temperature was slowly raised to  $60^{\circ}$ , a small amount of material with very little absorption at  $240\text{ m}\mu$  condensed and was discarded. At  $100^{\circ}$ , the desired substance was slowly deposited on the cold finger and was removed in four portions during the course of 3 hours, leaving a deeply colored viscous gum in the distilling flask. The four fractions exhibited similar specific absorptions at  $240\text{ m}\mu$  and were combined. The product, estimated to contain 450 mg. of the compound, was a colorless, odorless oil, which exhibited some cloudiness when chilled. Further purification was achieved by chromatographic adsorption on aluminum oxide. 450 mg. of material which had been distilled were dissolved in 10 cc. of dry ether or chloroform and drawn through a column of freshly ignited alumina, 1.8 cm. in diameter and 3 cm. high. The column was developed by a small volume of solvent and the desired substance was eluted by a large volume of the same solvent or by solvent containing 1.0 per cent of alcohol. When crude preparations were treated in this way, a large quantity of colored material remained on the column and was removed only by solvents with a higher alcohol content. Each small fraction of eluted material was chilled in an alcohol-dry ice mixture, whereupon one solidified. The mass was broken up in chilled methanol, and the fine crystals were collected by filtration at the temperature of dry ice.

*Determination of Thiooxazolidone in Plant Tissues*—The seed, root, or other portion of the plant was ground in a Waring blender with a measured volume of water and allowed to stand for several hours with occasional stirring. A portion was then heated in a boiling water bath and filtered. 1 cc. of the clear filtrate was shaken with 10 cc. of ether, and, after separation of phases was complete, 1 to 5 cc. of the ether was evaporated to dryness and the residue dissolved in 5 or 10 cc. of water. The ultraviolet absorption spectrum was plotted and the density at  $240\text{ m}\mu$  was corrected for non-specific absorption as follows. The spectrum of the pure substance exhibited a minimum at  $220\text{ m}\mu$ , at which wave-length the density was one-fifth of the density at  $240\text{ m}\mu$ ; also, the density at  $260\text{ m}\mu$  was one thir-



teenth that at 240  $m\mu$ . Furthermore, the density of non-specific absorption seemed to increase roughly linearly from 260 to 220  $m\mu$ . On this assumption, the corrected density at 240  $m\mu$  was calculated by subtracting from the observed density the average of the densities at 220 and 260  $m\mu$ , and multiplying the result by

$$= \frac{5 \times 13}{5 \times 13 - \frac{(5 + 13)}{2}} = \frac{65}{56}$$

The content was calculated from the corrected specific absorption and the dilution in extraction. It was assumed that the thiooxazolidone was distributed uniformly throughout the original mash, and that the partition ratio between water and ether was 1. Use of a 10-fold volume of ether minimized the importance of the latter assumption. This quantitative method is not specific, since related compounds would give similar absorption spectra.

*Properties*—The substance crystallized from ether as large, clear, colorless prisms; m.p. 50°;  $[\alpha]_D^{31} = -70.5^\circ$ ; (2 per cent in methanol; 51.6 mg. in 2.51 cc., 1 dm.  $-1.45^\circ$ ).

*Analysis*— $C_6H_7ONS$

Calculated. C 46.49, H 5.46, N 10.84, S 24.82, mol. wt. 129

Found. " 46.50, " 5.55, " 10.74, " 24.45, " " 121,  $CH_2-C < 0.1$

The molecular weight was kindly determined by Dr. Paul Bell of the American Cyanamid Company by a modification of the thermoelectric osmometer method (27, 28). The *C*-methyl analyses were performed on 50 to 100 mg. samples by the usual procedure (29); control experiments on 6-methyl-2-thiouracil and 5,5-dimethyl-2-thiooxazolidone yielded 0.8 terminal methyl each.

Ultraviolet absorption spectra were measured with a Beckman ultraviolet spectrophotometer, model DU. The analogues, 2-thiooxazolidone (30)<sup>2</sup> and 5,5-dimethyl-2-thiooxazolidone (31), were synthetic samples. The infra-red absorption spectrum in 2 per cent chloroform solution was obtained with a Baird infra-red recording spectrophotometer, model B, at Harvard University.

The dissociation constant was obtained by two methods. Calculations from the shift of ultraviolet absorption in various buffers yielded a pK of 10, whereas titration of an aqueous solution with 0.2 N NaOH with a Beckman type E glass electrode gave an uncorrected value of 10.5. The pK of 5,5-dimethyl-2-thiooxazolidone determined concurrently was found to be 11.05. The titration curves indicated an equivalent weight of 125 to 135; the end-point was not sharp.

*Distribution between Solvents*—The distribution constants of the turnip

factor between water and various organic solvents were measured spectrophotometrically at room temperature. The ratios (organic solvent to water) at an initial concentration of 5  $\gamma$  per cc. in water were ligroin 0.022, toluene 0.092, chloroform 1.29, ethyl acetate 2.52, and *n*-butanol 3.46. The ratios between ether and various aqueous solutions initially containing 0.104 mg. per cc. of the substance were water 0.86, 0.1 N HCl 0.85, saturated NaCl 2.20, 0.5 M NaHCO<sub>3</sub> 0.98, 0.5 M NaHCO<sub>3</sub> + 0.5 M Na<sub>2</sub>CO<sub>3</sub> 1.01, 0.5 M NaHCO<sub>3</sub> +  $\frac{1}{2}$  M Na<sub>2</sub>CO<sub>3</sub> 0.88, 0.5 M Na<sub>2</sub>CO<sub>3</sub> 0.20, and 0.1 N NaOH 0.02.

*Degradations*—A solution of 20 mg. of the turnip factor in 2 cc. of sodium hydroxide was boiled down to 0.5 cc. in 15 minutes. The ultraviolet spectrum was unchanged, and the compound was reisolated after acidification and extraction into ether.

Upon refluxing 100 mg. of the compound in 12 cc. of 4 N hydrochloric acid, hydrogen sulfide was evolved, and observation of the ultraviolet spectrum after 90 minutes showed that less than 0.3 mg. of the thiooxazolidone remained. Following evaporation *in vacuo*, the optical rotation of the residue was measured in solution in 1.5 cc. of water and 1.5 cc. of 1 N alkali in a 1 dm. tube. The observed values,  $+0.09^\circ$  and  $+0.07^\circ$  ( $[\alpha]_D = +1^\circ$  to  $2^\circ$ ), were small and practically identical, and presumably were caused by impurity in the starting material.

95 mg. of the compound were dissolved in 8 cc. of 4 N hydrochloric acid and boiled for 90 minutes. The solution was evaporated *in vacuo*, dissolved in water, rendered alkaline with sodium hydroxide, and distilled into 1.0 cc. of 0.1 N hydrochloric acid. Titration established that only 0.06 m.eq. of base had distilled (equivalent to only 0.08 molecular equivalent of the original material). Tests of the distillate with Nessler's solution showed that it contained less than 0.1 mg. of ammonia.

#### SUMMARY

An antithyroid compound, equal in potency to thiouracil in man, has been isolated from the root and seed of turnip and from the seed of cabbage, kale, and rape. This hitherto unknown substance was proved to be *l*-5-vinyl-2-thiooxazolidone.

We are indebted to Dr. R. O. Roblin and his associates of the Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, for helpful assistance.

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# THE UTILIZATION IN VITRO OF C<sup>14</sup>-LABELED ACETATE AND PYRUVATE BY DIAPHRAGM MUSCLE OF RAT\*

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The usefulness of the rat diaphragm technique of Gemmill in the study of carbohydrate metabolism of muscle *in vitro* has been demonstrated by the experiments of Gemmill (1, 2), Stadie (3, 4), Krahl and Cori (5), Krahl and Park (6), and Verzar and Wenner (7, 8). The adaptation of this technique to experiments by use of C<sup>14</sup>-labeled glucose was described in a previous paper (9). In those experiments, it was found that the addition of insulin increased the amount of glucose utilized, the amount of glycogen synthesized, and the amount of glucose carbon metabolized to carbon dioxide. The removal of the pituitary and adrenals also increased the rates of these reactions. Although most of the data could be explained by assuming that insulin and the hormones of the pituitary and adrenal glands affect the rate of the hexokinase reaction, other points of effect by these substances were not excluded. In the experiments reported here, the effect of insulin on the metabolism of C<sup>14</sup>-labeled acetate and pyruvate was studied under varying experimental conditions. Other experiments comparing the utilization of labeled acetate and pyruvate in ventricle slices and diaphragm of the rat are reported elsewhere (10).

## Materials and Methods

The strain of rats and methods of adrenalectomy and of inducing diabetes were identical with those of the previous study (9). Rats were fasted 24 hours before being used in an experiment, and only those rats with a fasting blood glucose of 300 mg. per cent or more were included in the diabetic series.

Sodium acetate labeled with C<sup>14</sup> in the carboxyl carbon (11) and potassium pyruvate labeled with C<sup>14</sup> in the carbonyl carbon (12) were synthesized in this laboratory by Dr. Yale J. Topper. The radioactivities of these compounds were determined by their combustion to CO<sub>2</sub> and collection as BaCO<sub>3</sub>. The BaCO<sub>3</sub> was plated on stainless steel planchets and its radioactivity determined by an end window Geiger tube. The acetate had an activity of 3110 counts per minute per mg. when referred to an arbitrary

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standard of  $\text{BaC}^{14}\text{O}_3$ ; the pyruvate had an activity of 1465 counts per minute per mg. when referred to the same standard.

The isolated hemidiaphragms were incubated in Warburg vessels in a medium of 0.04 M sodium phosphate, 0.005 M  $\text{MgCl}_2$ , 0.08 M NaCl, and either 0.01 M acetate or 0.01 M pyruvate. The initial pH of the medium, determined by glass electrode, was 6.8 and the pH after incubation was  $6.8 \pm 0.1$ . The center wells contained a piece of hard filter paper and 0.2 ml. of  $\text{CO}_2$ -free 5 per cent NaOH to absorb the  $\text{CO}_2$  produced. A hemidiaphragm weighing about 100 mg. was placed in each vessel in 3.0 ml. of medium, 0.5 unit of insulin per ml. was added to one of each pair of flasks, and the vessels were incubated for 2 hours after an initial gassing with 100 per cent oxygen.

At the end of the incubation period, the  $\text{CO}_2$  from the center well was precipitated as  $\text{BaCO}_3$ , plated, and counted with an end window Geiger tube. The diaphragm was removed and digested in boiling 30 per cent KOH; the glycogen was precipitated by alcohol, purified, hydrolyzed, and determined as glucose by the method of Nelson (13). Samples of the media were analyzed before and after incubation for pyruvate by the method of Friedemann and Haugen (14) and for lactate by the method of Barker and Summerson (15). Blood glucose determinations were made at the time of sacrifice by the method of Nelson (13).

### Results

*Acetate Metabolism*—The amount of acetate oxidized to  $\text{CO}_2$  was calculated from the ratio of the total activity in the respiratory  $\text{CO}_2$  to the total initial activity of the acetate in the medium, multiplied by the initial concentration of acetate in the medium (Table I, Line 2). This calculation involves the assumption that the formation of radioactive  $\text{CO}_2$  is a measure of the complete oxidation of acetate molecules, and that for each labeled (carboxyl) carbon appearing in the  $\text{CO}_2$  1 unlabeled ( $\alpha$ ) carbon also appears.

The metabolism of acetate to  $\text{CO}_2$  in diabetic diaphragm was much less than that in normal diaphragm (Table I, Line 2),  $3.3 \mu\text{M}$  per gm. wet weight of diaphragm per hour in muscle from diabetic animals compared to  $12.4 \mu\text{M}$  per gm. per hour in muscle from normal rats. This was not due to an over-all decrease in metabolism in the diabetic tissue, because the oxygen consumptions of the two types of tissue are comparable (Table I, Line 5). Rather, it is to be attributed to an actual decrease in the diabetic muscle of the percentage of the  $\text{CO}_2$  carbons derived from acetate (Table I, Line 6). The percentage of  $\text{CO}_2$  carbons derived from acetate carbons was calculated by dividing the specific activity (counts per minute per millimole of carbon) of the  $\text{CO}_2$  by the specific activity (counts per minute per

millimole of carbon) of the acetate and multiplying by 100. This decrease in acetate metabolism in the diabetic was not relieved by the addition *in vitro* of insulin. The amount of acetate metabolized to carbon dioxide and the percentage of respiratory  $\text{CO}_2$  derived from acetate in diaphragm from adrenalectomized rats was not significantly different from normal (Table I). Insulin had no effect on acetate metabolism in muscle from either normal or adrenalectomized rats. This is in striking contrast to its marked effect (when glucose is the substrate) on glucose utilization, glycogen formation, and the metabolism of glucose carbons to carbon dioxide (9).

There was no net increase in the amount of glycogen present in diaphragm incubated with acetate as the substrate. The amount of lactate

TABLE I

*Effect of Insulin on Metabolism of Acetate by Diaphragm Muscle*

The figures given are the mean  $\pm$  the standard error in micromoles per gm. per hour, except for respiratory  $\text{CO}_2$  which is in per cent.

	Normal rat diaphragm		Diabetic rat diaphragm		Adrenalectomized rat diaphragm	
	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin
1. No. of experiments	7	7	8	8	9	9
2. Acetate oxidized	12.4 $\pm 0.53$	12.9 $\pm 0.60$	3.3 $\pm 0.47$	3.8 $\pm 0.58$	11.1 $\pm 1.3$	11.1 $\pm 1.5$
3. Lactate produced	4.4 $\pm 0.98$	5.7 $\pm 0.72$	4.6 $\pm 0.47$	6.0 $\pm 1.01$	1.6 $\pm 0.51$	2.2 $\pm 0.33$
4. Glycogen made	0	0	0	0	0	0
5. Oxygen uptake	58.2 $\pm 2.0$	61.6 $\pm 2.0$	55.4 $\pm 2.1$	55.6 $\pm 1.6$	51.5 $\pm 2.4$	57.8 $\pm 1.6$
6. Respiratory $\text{CO}_2$ derived from acetate	42.2 $\pm 2.1$	43.6 $\pm 2.9$	15.4 $\pm 4.0$	16.2 $\pm 1.8$	40.4 $\pm 3.6$	43.3 $\pm 3.7$

produced by diaphragm muscle from adrenalectomized rats was less than half that produced by muscle from normal or diabetic animals (Table I, Line 3).

**Pyruvate Metabolism**—The metabolism of carbonyl-labeled pyruvate to  $\text{CO}_2$  by muscle from diabetic rats was also significantly lower than that of normal diaphragm, but, in contrast to acetate metabolism, it was brought back to normal by the addition, *in vitro*, of insulin (Table II, Line 3). The amount of pyruvate metabolized to  $\text{CO}_2$  was calculated by dividing the total activity in the respiratory  $\text{CO}_2$  by the total activity of the pyruvate in the medium and multiplying by the initial concentration of pyruvate in the medium. This calculation involves the assumption that the formation of radioactive  $\text{CO}_2$  is a measure of the complete oxidation of pyruvate

molecules, and that, for each labeled (carbonyl) carbon appearing in the  $\text{CO}_2$ , 2 unlabeled ones (a carboxyl and a methyl carbon) also appear as  $\text{CO}_2$ .

The total utilization of pyruvate, measured chemically as the disappearance of pyruvate from the medium, was also somewhat lower in diabetic diaphragm, and this was restored to normal by the addition *in vitro* of insulin (Table II, Line 2). In contrast, insulin caused no significant increase in either the total utilization of pyruvate or the metabolism of pyruvate to  $\text{CO}_2$  in normal or adrenalectomized diaphragm (Table II). The percentage of the respiratory  $\text{CO}_2$  derived from pyruvate was not de-

TABLE II

*Effect of Insulin on Metabolism of Pyruvate by Diaphragm Muscle*

The figures given are the mean  $\pm$  the standard error in micromoles per gm. per hour, except for respiratory  $\text{CO}_2$  which is in per cent.

	Normal rat diaphragm		Diabetic rat diaphragm		Adrenalectomized rat diaphragm	
	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin
1. No. of experiments	10	10	7	7	7	7
2. Total pyruvate utilization	42.4	44.3	32.1	45.7	39.1	39.8
	$\pm 2.1$	$\pm 2.5$	$\pm 3.8$	$\pm 4.8$	$\pm 2.9$	$\pm 3.1$
3. Pyruvate oxidized	8.5	8.8	6.3	9.8	11.8	12.3
	$\pm 0.38$	$\pm 0.50$	$\pm 0.60$	$\pm 1.1$	$\pm 0.68$	$\pm 0.70$
4. Glycogen made	1.23	0.87	0.99	0.13	1.58	1.55
	$\pm 0.10$	$\pm 0.30$	$\pm 0.98$	$\pm 0.11$	$\pm 0.63$	$\pm 0.54$
5. Lactate produced	18.5	17.7	22.1	25.1	12.3	9.3
	$\pm 2.7$	$\pm 1.8$	$\pm 2.8$	$\pm 1.6$	$\pm 0.43$	$\pm 1.2$
6. Oxygen uptake	63.2	65.0	48.4	55.7	59.6	62.4
	$\pm 2.9$	$\pm 3.3$	$\pm 4.3$	$\pm 4.1$	$\pm 2.0$	$\pm 1.3$
7. Respiratory $\text{CO}_2$ derived from pyruvate	34.8	35.1	35.4	41.3	43.7	45.6
	$\pm 1.4$	$\pm 1.6$	$\pm 3.2$	$\pm 1.0$	$\pm 2.4$	$\pm 2.1$

creased in the diabetic muscle (Table II, Line 7), in contrast to the marked decrease in the percentage of respiratory  $\text{CO}_2$  derived from acetate in diaphragms of diabetic animals.

Although the over-all utilization of pyruvate was not increased in the muscle from adrenalectomized animals (Table II, Line 2), the rate of metabolism of pyruvate to  $\text{CO}_2$  was increased significantly (Table II, Line 3), and hence the fraction of the pyruvate utilized that is metabolized to  $\text{CO}_2$  was increased. In normal and diabetic diaphragms, approximately 20 per cent of the pyruvate disappearing from the medium was metabolized to  $\text{CO}_2$ , but in muscle from adrenalectomized rats 30 per cent of the pyruvate disappearing from the medium was metabolized to  $\text{CO}_2$ . The amount

of lactate produced by adrenalectomized muscle was significantly less than that produced by normal or diabetic muscle when pyruvate was the substrate.

The amount of glycogen produced by diaphragm muscle incubated with pyruvate as a substrate was slight and variable (Table II, Line 4). There were no significant differences in this respect between normal, diabetic, or adrenalectomized muscle, or between muscle incubated with or without insulin.

TABLE III

*Effect of Added Dicarboxylic and Tricarboxylic Acids on Acetate Metabolism in Muscle*

The figures given are the mean  $\pm$  the standard error.

	No. of experiments	Acetate oxidized, $\mu$ M per gm. per hr.		Oxygen uptake, $\mu$ M per gm. per hr.		Respiratory CO <sub>2</sub> from acetate	
		Without addition	With addition	Without addition	With addition	Without addition	With addition
Normal muscle							
						<i>per cent</i>	<i>per cent</i>
1. Aconitate, 20 mm per liter		10.4	11.8	66.2	72.4	34.1	32.8
		$\pm 0.74$	$\pm 0.89$	$\pm 2.3$	$\pm 3.0$	$\pm 3.0$	$\pm 2.4$
2. $\alpha$ -Ketoglutarate, 20 mm per liter		8.2	10.6	66.0	73.8	29.4	30.3
		$\pm 0.88$	$\pm 0.80$	$\pm 1.6$	$\pm 2.4$	$\pm 1.8$	$\pm 3.3$
Oxalacetate, 10 mm per liter	4	8.5	9.3	61.0	69.4	31.8	13.0
		$\pm 0.82$	$\pm 0.87$	$\pm 2.2$	$\pm 2.9$	$\pm 2.4$	$\pm 1.9$
Diabetic muscle							
4. Aconitate, 20 mm per liter	4	4.9	11.1	50.4	61.2	26.3	30.3
		$\pm 0.71$	$\pm 0.77$	$\pm 3.2$	$\pm 2.5$	$\pm 2.2$	$\pm 2.5$
5. $\alpha$ -Ketoglutarate, 20 mm per liter	2	5.1	7.3	69.6	74.6	22.2	21.6
		$\pm 0.80$	$\pm 0.91$	$\pm 4.3$	$\pm 3.6$	$\pm 1.7$	$\pm 1.9$
6. Oxalacetate, 5 mm per liter	5	4.2	8.6	52.6	65.0	21.7	16.2
		$\pm 0.84$	$\pm 0.72$	$\pm 2.3$	$\pm 2.9$	$\pm 2.0$	$\pm 1.4$

The effects of the addition of certain Krebs tricarboxylic acid cycle intermediates on the metabolism of labeled acetate and pyruvate were also studied. Both aconitate and  $\alpha$ -ketoglutarate (20 mm per liter) produced slight increases in the amount of acetate metabolized to CO<sub>2</sub> by normal muscle, but since they also increased the over-all metabolism (as measured by oxygen consumption), there was no effect on the percentage of the respiratory CO<sub>2</sub> derived from the labeled acetate (Table III). The addition of aconitate to the incubation medium increased markedly the amount of acetate metabolized to CO<sub>2</sub> by diabetic diaphragm and brought



the value up to the normal level (Table III, Line 4). As in normal muscle, the addition of aconitate increased the oxygen consumption of muscle from diabetic rats, and hence the increase in the percentage of respiratory  $\text{CO}_2$  derived from acetate was slight. In contrast, added  $\alpha$ -ketoglutarate had less effect on acetate metabolism in diabetic diaphragm.

Added aconitate (20 mm per liter) produced a marked increase in the amount of pyruvate utilized (from 49 to 60  $\mu\text{M}$  per gm. per hour) (Table IV). There was also observed an increase in the amount of pyruvate

TABLE IV

*Effect of Added Dicarboxylic and Tricarboxylic Acids on Pyruvate Metabolism in Muscle*

The figures given are the mean  $\pm$  the standard error.

	No. of experiments	Total pyruvate utilized, $\mu\text{M}$ per gm. per hr.		Pyruvate oxidized, $\mu\text{M}$ per gm. per hr.		Oxygen uptake, $\mu\text{M}$ per gm. per hr.		Respiratory $\text{CO}_2$ from pyruvate	
		Without addition	With addition	Without addition	With addition	Without addition	With addition	Without addition	With addition
Normal muscle									
1. Aconitate, 20 mm per liter	4	49.0 $\pm 2.6$	60.3 $\pm 4.9$	9.1 $\pm 0.68$	16.1 $\pm 0.99$	71.2 $\pm 1.9$	85.8 $\pm 5.6$	35.2 $\pm 3.3$	46.7 $\pm 1.6$
2. $\alpha$ -Ketoglutarate, 20 mm per liter	4	46.6 $\pm 2.2$		9.3 $\pm 0.76$	10.7 $\pm 1.3$	78.2 $\pm 0.4$	76.7 $\pm 3.5$	32.2 $\pm 1.8$	32.3 $\pm 1.9$
3. Succinate, 10 mm per liter	2	39.1 $\pm 3.1$	40.4 $\pm 2.8$	7.3 $\pm 0.66$	8.3 $\pm 0.74$	70.5 $\pm 2.2$	74.0 $\pm 5.0$	32.1 $\pm 2.5$	31.7 $\pm 1.9$
4. Oxalacetate, 5 mm per liter	4	49.9 $\pm 3.3$		8.5 $\pm 0.81$	7.4 $\pm 0.71$	68.2 $\pm 3.1$	70.8 $\pm 3.4$	32.8 $\pm 1.8$	17.4 $\pm 1.5$
Diabetic muscle									
5. Aconitate, 20 mm per liter	4	43.9 $\pm 4.6$	55.2 $\pm 4.5$	6.8 $\pm 1.3$	9.4 $\pm 1.3$	59.0 $\pm 5.1$	68.0 $\pm 3.9$	29.9 $\pm 4.5$	34.1 $\pm 3.3$
6. Oxalacetate, 5 mm per liter	3	24.4 $\pm 3.1$		3.1 $\pm 0.52$	3.5 $\pm 0.58$	58.8 $\pm 4.7$	60.8 $\pm 5.2$	16.1 $\pm 1.5$	9.6 $\pm 1.1$

metabolized to  $\text{CO}_2$  (from 9 to 16  $\mu\text{M}$  per gm. per hour), and in the percentage of respiratory  $\text{CO}_2$  derived from pyruvate. In contrast,  $\alpha$ -ketoglutarate produced only a slight, statistically insignificant increase in the amount of pyruvate metabolized to  $\text{CO}_2$  and no change in the percentage of respiratory  $\text{CO}_2$  derived from pyruvate. Neither aconitate nor  $\alpha$ -ketoglutarate increased the amount of lactate produced with either pyruvate or acetate as the substrate.

Oxalacetate had no effect on the amount of acetate or pyruvate metabolized to carbon dioxide in normal muscle (Tables III and IV) but, be-

cause it was rapidly decarboxylated and produced a large amount of carbon dioxide, diluting that from the metabolism of the acetate and pyruvate, the percentage of the respiratory  $\text{CO}_2$  derived from acetate and pyruvate was markedly decreased in these experiments. Oxalacetate increased the amount of acetate, but not of pyruvate, metabolized to carbon dioxide by diabetic diaphragm.

#### DISCUSSION

These experiments indicate that, in alloxan diabetes of the rat, the overall utilization of pyruvate and its metabolism to  $\text{CO}_2$  by diaphragm muscle are interfered with, and that this condition can be remedied by the addition *in vitro* of insulin. This suggests that insulin is involved in the metabolism of pyruvate to  $\text{CO}_2$ . The experiments comparing metabolism in cardiac and skeletal muscle of the rat (10), in which slightly different experimental techniques were used, also showed decreases in the metabolism of acetate and pyruvate to  $\text{CO}_2$  in diabetic as compared to normal diaphragm. These investigators also found that the addition of insulin *in vitro* would increase in diabetic but not in normal diaphragm the amount of pyruvate but not of acetate metabolized to carbon dioxide. These findings are in agreement with the experiments of Shorr (16) who found that heart muscle slices from depancreatized diabetic dogs showed a decreased utilization of pyruvate and lactate, and with the results of Rice and Evans (17) who demonstrated an effect of insulin on pyruvate utilization by pigeon breast muscle mince. An effect of insulin in increasing the incorporation of pyruvate into lipide by rat diaphragm muscle has also been observed (18,19). Further evidence that insulin may be involved in the metabolism of pyruvate is supplied by the experiments of Charalampous and Hegsted (20) who found that acetylation, as measured by the acetylation of injected *p*-aminobenzoic acid, was decreased in alloxan-diabetic rats and brought back to normal by the injection of insulin. Charalampous and Hegsted also found that acetylation in the diabetic was increased by the injection of adenosine triphosphate, acetyl phosphate, and certain di- and tricarboxylic acids, of which malate was most effective. None of the materials effective in diabetic rats had any effect on acetylation when injected in normal rats. This suggests that insulin is involved in the metabolism of pyruvate in the Krebs cycle, since the injection of Krebs cycle components, but not of lactate or pyruvate, increased the amount of acetylation in diabetic animals.

Our experiments show that alloxan diabetes of the rat is also characterized by a marked reduction in the metabolism of acetate to carbon dioxide. This reduction is unchanged when insulin is added *in vitro*. The experiments on diabetic diaphragm could be accounted for by interference with

the condensation of acetate and oxalacetate in the Krebs cycle. Since the addition of insulin to diabetic diaphragm did not restore the metabolism of acetate to normal, it is probable that insulin deficiency *per se* was not responsible for the diminished acetate metabolism.

We wish to express our thanks to Dr. A. K. Solomon of the Biophysical Laboratory for performing the  $C^{14}$  analyses, and to Miss Kathleen Whitehouse and Mrs. Vivien White for their technical assistance.

#### SUMMARY

1. The metabolism of acetate and pyruvate to carbon dioxide, the synthesis of glycogen, the accumulation of lactic acid, and the disappearance of pyruvate have been measured *in vitro* in diaphragm muscle isolated from normal, diabetic, and adrenalectomized rats.

2. The amount of acetate metabolized to carbon dioxide is much less in muscle from diabetic rats than in muscle from normal rats. This decrease is not restored by the addition of insulin. The metabolism of acetate to carbon dioxide in muscle from adrenalectomized rats was at the normal level and was unaffected by insulin.

3. The amount of pyruvate metabolized to carbon dioxide was also less in muscle from diabetic animals than in normal muscle, but this was restored to normal by the addition *in vitro* of insulin. The total utilization of pyruvate was also decreased in diabetic muscle and brought back to normal when insulin was added. Insulin caused no significant increase in either the total utilization of pyruvate or its metabolism to carbon dioxide in muscle from normal or adrenalectomized rats.

4. Adrenalectomy produced an increase in the fraction of the total pyruvate utilized that is metabolized to carbon dioxide and a decrease in the accumulation of lactate.

5. The effects of the addition of certain intermediates in the Krebs tricarboxylic acid cycle on the metabolism of acetate and pyruvate by diaphragm muscle from normal and diabetic rats were also studied.

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# A POLAROGRAPHIC MICROMETHOD FOR THE DETERMINATION OF BLOOD CHLORIDE\*

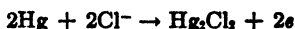
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Because of the inherent sensitivity of the dropping mercury electrode when used for the quantitative analysis of inorganic ions, the present study was undertaken to determine whether sufficient accuracy and simplicity of operation could be attained to justify its employment as a micromethod for the determination of chloride in blood. Since it was desired to work with such samples as can be conveniently obtained from small laboratory animals for serial analyses, a method has been developed for 0.02 and 0.05 ml. samples of whole blood, plasma, and serum.

Kolthoff and Miller (1) have shown that the concentration of chloride ion in a solution can be determined by measurement of the diffusion current of the anodic depolarization wave produced at the dropping mercury electrode. Using 0.1 M potassium nitrate as supporting electrolyte, they found that the diffusion current was directly proportional to chloride concentrations between  $10^{-4}$  and  $2 \times 10^{-3}$  equivalent per liter. The electrode reaction is



In applying their findings to the development of a method for the determination of chloride in blood, the general procedure used for the preparation of the sample has been to remove proteins, provide a supporting electrolyte, and dilute the sample about 1:100 in as few operations as possible. Phosphotungstic acid is used for the dual purpose of precipitating proteins and of acting as a supporting electrolyte. The concentration of chloride in the clear supernatant solution obtained on centrifugation is determined by a single measurement of the diffusion current at a fixed applied voltage.

## Apparatus

A manual apparatus similar to that described by Kolthoff and Lingane (2) was used in the determinations described in this report. A Leeds and Northrup student model potentiometer was used to set the potential of the

\* A preliminary report was presented before the American Society of Biological Chemists at Detroit, April, 1949.

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dropping electrode. The diffusion current was measured with a reflecting galvanometer and scale. The sensitivity of the galvanometer was adjusted to approximately  $0.013 \mu\text{a. per mm.}$ , and the instrument was overdamped to reduce the amplitude of the current oscillations. The drop times of the capillaries used were between 2 and 4 seconds.

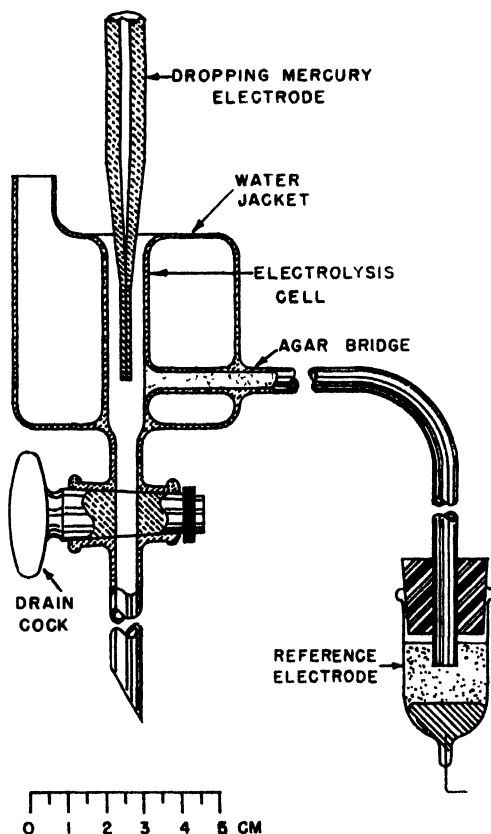


FIG. 1. Electrolysis cell-reference electrode assembly

A compact electrolysis cell-reference electrode assembly was devised which contributed greatly to the practicability of the method for routine use (Fig. 1). The assembly consists of a saturated mercurous sulfate reference electrode (+0.4 volt *versus* saturated calomel electrode), an electrolysis cell of about 1 ml. capacity, and a dropping mercury electrode, all aligned permanently on a ring-stand. The cell is drained by means of the stop-cock at the bottom; thus it need not be removed for filling or rinsing. A water jacket containing about 100 ml. encloses the electrolysis cell to

minimize the effect of any change in room temperature during a series of analyses. It is not necessary to know the temperature of the cell, since the unknown solutions and standards are compared at the same temperature.

*Reagents—*

1. Phosphoric acid solution, approximately 0.15 M. Into a 1 liter volumetric flask transfer 10 ml. of phosphoric acid (sp. gr. 1.7) and dilute to the mark with distilled water.

2. Sodium tungstate solution, 3.0 per cent. Dissolve 30 gm. of reagent grade sodium tungstate in distilled water and dilute to 1 liter.

3. Stock standard potassium chloride solution, 0.2 M. Dissolve 14.91 gm. of dried, analytical reagent grade potassium chloride in distilled water and dilute to 1 liter.

4. Diluted standard potassium chloride solutions. Transfer to 100 ml. volumetric flasks 55, 50, 45, 40, and 35 ml. of the stock standard potassium chloride solution. Dilute to 100 ml. to prepare standards containing 110, 100, 90, 80, and 70 m.eq. of chloride per liter, respectively.

5. Working standard solutions. To 100 ml. of a phosphotungstic acid solution, prepared from 80 ml. of the approximately 0.15 M phosphoric acid solution and 20 ml. of the 3 per cent sodium tungstate solution, add 1.0 ml. of one of the diluted standard potassium chloride solutions. Since the dilution of the chloride standards in these working standard solutions is the same as the dilution of the blood samples, each will correspond to a chloride concentration in the blood sample equivalent to the chloride concentration of the standard solution from which it was prepared.

*Procedure*

The following technique is used in the preparation of 0.05 ml. samples of whole blood, serum, or plasma.<sup>1</sup> The sample is delivered into 4.0 ml. of the approximately 0.15 M phosphoric acid in a test-tube, and 1.0 ml. of the 3 per cent sodium tungstate solution is added to precipitate proteins. The solution is thoroughly mixed and then centrifuged approximately 1 minute at 2000 R.P.M. The clear supernatant solution is then ready to be transferred to the electrolysis cell.

When 0.02 ml. samples are used, the procedure is exactly the same except

<sup>1</sup> The whole blood is drawn directly from the animal into the pipette and delivered into the diluting solution, before clotting can occur, to obviate the necessity for an anticoagulant. For serum determinations the blood is drawn into a soft glass capillary tube, and one end of the tube is sealed in a Bunsen flame at some distance from the blood column (to prevent rupture of red cells). After centrifugation the capillary is scratched with a diamond pencil at the junction of the serum and red cells. The tube is broken off and the serum drawn directly into a pipette from the capillary.



that the quantities of 0.15 M phosphoric acid and 3 per cent sodium tungstate used are reduced to 1.6 and 0.4 ml., respectively.

Since it is unnecessary to record complete current-voltage curves for routine analyses, a polarimetric procedure is used; *i.e.*, the current is measured at a single, fixed, applied voltage. A voltage within the diffusion current of the chloride wave is chosen at which increments in chloride concentration produce directly proportional increments in current. For the procedure described here, a setting of  $-0.06$  volt against the saturated mercurous sulfate reference electrode was found suitable ( $+0.34$  volt *versus* saturated calomel electrode). Oxygen need not be removed from the solutions to be analyzed, since it is reduced at potentials negative to the chloride wave.

The cell is rinsed once with the solution to be analyzed, refilled, and the minimal and maximal galvanometer readings are recorded. A sufficient number of the standard chloride solutions in phosphotungstic acid to cover the range of chloride values encountered in the material used are run simultaneously. The mean deflections for the blood dilutions are referred to the calibration provided by the mean deflections for the chloride standards.

#### EXPERIMENTAL

The accuracy of the method was evaluated by the following studies.

1. Calibration curves show a direct proportionality of diffusion current to chloride ion concentration.

2. The precision of the method as shown by the reproducibility of galvanometer readings with successive samples of the same chloride solution is generally within 1 scale division. At the usual setting of the galvanometer shunt a variation of 1 scale division amounts to 0.33 m.eq. per liter. In several series of ten successive samples of the same blood dilution it was found that the greatest difference in current readings within each series was not more than 2 scale divisions.

3. The agreement of duplicates was essentially the same whether the determinations were made on whole blood, serum, or plasma. A series of thirty-four pairs of 0.05 ml. samples showed an average difference between duplicates of 0.55 per cent, the greatest difference encountered being 1.5 per cent. Thirty-one pairs of 0.02 ml. samples showed an average difference of 0.75 per cent, the greatest difference, with one exception, being 1.5 per cent.

4. In nine experiments in which the equivalent of 16.0 m.eq. per liter of chloride as potassium chloride was added to 0.05 ml. samples of plasma and whole blood the mean error in recovery was 0.9 m.eq. per liter; the maximal error was 1.5 m.eq. per liter.

5. The values obtained by this method applied to 0.05 ml. samples of serum, plasma, and whole blood were compared with the values obtained

by the iodometric titration method of Sendroy, as modified by Van Slyke and Hiller (3), with 1 ml. samples. A series of twenty-eight parallel determinations in duplicate was carried out. The mean deviation between the values obtained by the two methods was 1.0 m.eq. per liter. 86 per cent of the polarographic values agreed with Sendroy values within 2.0 m.eq. per liter, and none differed by as much as 3.0 m.eq. per liter.

#### DISCUSSION

The foregoing study of the accuracy of this method indicates that it is adequate for following changes in blood chloride concentration. The pro-

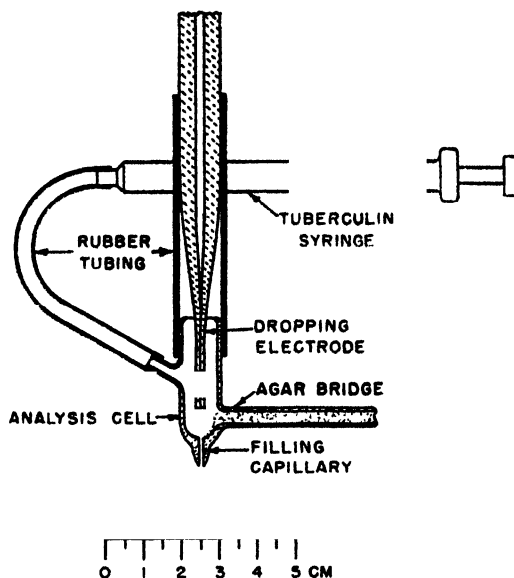


FIG. 2. Small electrolysis cell

cedure is simple and rapid, since preparation of the blood consists merely in the measurement and dilution of the sample, followed by centrifugation to remove the protein precipitate. The use of the polarimetric technique (a single current reading at a fixed applied voltage) and of the electrolysis cell assembly illustrated here contributes further to the simplicity of the method in routine use. Thus this method differs from the established volumetric micromethods in that a single galvanometer reading is substituted for the usual microtitration of an aliquot with a standardized solution.

By the use of a very small electrolysis cell this method could be adapted to the determination of much smaller quantities of chloride than those used here, with no other modification of the technique. To illustrate this a small electrolysis cell (Fig. 2) was devised which permitted the determination of

chloride concentration in 0.05 ml. of the phosphotungstic acid dilution of chloride. Calibration curves obtained with 0.05 ml. portions of 5, 7, 9, and  $11 \times 10^{-4}$  M chloride in phosphotungstic acid solution showed a direct proportionality between diffusion current and chloride concentration. The quantity of chloride ion in these samples was 0.89, 1.24, 1.60, and 1.95  $\gamma$ , respectively. With these small samples it was noted that the galvanometer reading remained constant at its maximal deflection for only about four current oscillations, and then drifted to lower values. The diffusion current readings for successive samples of the same solution generally agreed within 2 scale divisions (about 0.01  $\gamma$  of chloride).

While the above investigations were in progress, the work of Schönholzer (4) on the polarographic determination of chlorides in biological materials came to the attention of the authors. His technique is similar to that presented in this paper in that the chloride concentration in the blood dilution is determined by measurement of the diffusion current. However, complete current-voltage curves are recorded for each sample, and in this procedure the proteins are not removed from the solution to be analyzed. The sample is merely diluted in 0.1 N sulfuric acid, which acts as the supporting electrolyte. The accuracy of the procedure is stated to be  $\pm 2$  per cent. Because of the simplicity of this method of preparing the sample, its reproducibility and accuracy were studied with the apparatus and technique described in this paper. The reproducibility of galvanometer readings with successive samples of the same dilution of whole blood or plasma was the same as that found for phosphotungstic acid dilutions. A series of determinations in duplicate was carried out on nine whole bloods and nine serums with 0.05 ml. samples of both the sulfuric and phosphotungstic acid dilutions, together with parallel determinations on the same samples by the Sendroy macromethod (1 ml. samples). The mean deviation from the Sendroy values when sulfuric acid was used as the diluting fluid was 1.5 m.eq. per liter; with the phosphotungstic acid the mean deviation was 0.9 m.eq. per liter. The slightly better agreement of the latter procedure with the macromethod thus was not remarkable, even though on a theoretical basis it would seem better practice to make the blood dilutions as nearly like the chloride solutions used for calibration as possible by the removal of proteins.

There are a number of substances, not normally present in blood or present only in inappreciable quantities, which also depolarize the dropping mercury electrode at the voltage used in the determination of chloride. The only such substances likely to be encountered in experimental work are thiosulfate, thiocyanate, bromide, and iodide. When any of these is present, its diffusion current will be added to that of the chloride at  $-0.06$  volt *versus* the saturated mercurous sulfate electrode.

The presence of such interfering substances can be detected readily by

comparing the current of the blood dilution at a potential just negative to the beginning of the chloride wave ( $-0.2$  volt *versus* the saturated mercurous sulfate electrode, or  $+0.2$  volt *versus* the saturated calomel electrode) with that of one of the standard chloride solutions or of a blank solution of phosphotungstic acid. In the absence of interfering ions the current will be identical for all three solutions at this voltage; the presence of an interfering ion in the diluted blood will give rise to a current greater than that of a standard chloride solution or a blank. However, such interfering substances are not likely to be encountered in blood unless they have been purposely administered, and for practical routine work the procedure is much more rapid when only a single current measurement is made.

#### SUMMARY

A method has been developed for the determination of chloride in 0.02 and 0.05 ml. samples of blood by means of polarimetric analysis with the dropping mercury electrode. It is rapid, simple, and accurate.

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# A SUBMICRODETERMINATION OF GLUCOSE\*

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The reduction of ferricyanide ions in alkaline solution followed by formation of Prussian blue (ferric ferrocyanide), which is measured quantitatively, has been the basis of a method for the estimation of blood sugar (1). This method has been modified several times (2-6). Though the reduction reaction suffers from lack of specificity, the convenience and sensitivity of the procedure often make it the method of choice, especially when the reducing materials in the system are known. The colorimetric method of Folin and Malmros (3), as modified by Horvath and Knehr (5), is perhaps the most convenient modification. The Prussian blue is held in suspension by Duponol<sup>1</sup> and is measured with the aid of a photoelectric colorimeter. The range of the method is 20 to 140  $\gamma$  of glucose.

Occasionally a more sensitive method is preferable. In this paper, a modification of the ferricyanide reduction method, which has a range of 1 to 9  $\gamma$  of glucose, is described.

## *Description of Method*

### *Reagents—*

1. Ferricyanide solution. 0.5 gm. of potassium ferricyanide per liter; stored in a brown bottle.
2. Carbonate-cyanide solution. 5.3 gm. of sodium carbonate + 0.65 gm. of KCN per liter.
3. Ferric iron solution. 1.5 gm. of ferric ammonium sulfate + 1 gm. of Duponol in 1 liter of 0.05 N sulfuric acid.

### *Procedure*

The sample, deproteinized and neutralized if necessary and containing 1 to 9  $\gamma$  of glucose, is placed in an 18 mm. Pyrex test-tube and diluted to volume (1 to 3 ml. as desired). 1 ml. each of carbonate-cyanide solution and of ferricyanide solution is added. After mixing, the tube is heated

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<sup>1</sup> Duponol ME dry (sodium monolauryl sulfate) obtained from E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

in a boiling water bath for 15 minutes. 5 ml. of ferric iron solution are mixed with the sample after cooling. 15 minutes are allowed for color development. The sample is then read against a reagent blank in a photo-electric colorimeter at 690  $m\mu$  (any wave-length between 650 and 730  $m\mu$  is suitable). A standard glucose sample is run in parallel. Calculation of

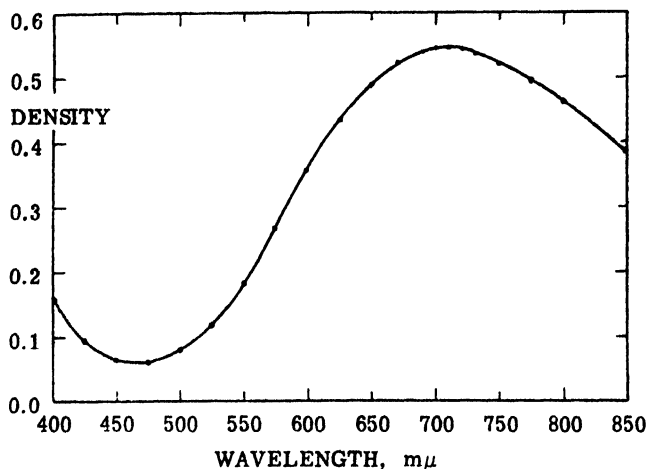


FIG. 1. Absorption spectrum of Prussian blue

TABLE I  
*Glucose Recovery in Replicate Analyses*

Glucose added	Glucose recovered*		
	Sample 1	Sample 2	Sample 3
$\gamma$	$\gamma$	$\gamma$	$\gamma$
0.90	1.05	1.09	0.97
1.80	1.82	1.83	1.66
2.70	2.64	2.66	2.57
3.60	3.50	3.33	3.40
5.40	5.40	5.33	
7.20	7.00	7.18	
9.00	8.95	9.05	

\* Assuming extinction of 0.118 = 1  $\gamma$  of glucose.

the reducing sugar in the unknown sample is made on the assumption that Beer's law holds.

Because of the sensitivity of the method, it is best to reserve a set of test-tubes for use only in the determination; the tubes are cleaned immediately after use by rinsing with distilled water.

## DISCUSSION

The absorption spectrum of Prussian blue is given in Fig. 1. As can be seen, greatest absorption occurs in the region near 700  $m\mu$ . The sensitivity was increased over 7-fold by using light in this region rather than at 490  $m\mu$  as recommended by Horvath and Knehr. Since the final volume was reduced, the quantity of sample was proportionately decreased, so that an over-all reduction in sample size of about 20-fold was achieved.

The quantities of reagents used were chosen so as to effect rapid reduction and rapid color development. The rate of reduction is affected by cyanide. Without cyanide, the reduction is only half completed in 15 minutes, but with 500  $\gamma$  or more of KCN per tube the reaction is complete in less than 15 minutes. The quantity of KCN added in this procedure is 650  $\gamma$ . The quantity of Duponol used is near the minimum effective amount, since with higher concentrations of Duponol air bubbles tend to remain suspended in the solution.

The principal advantage of this method is its greater sensitivity. In addition, the relation between sugar and extinction follows Beer's law which, as Horvath and Knehr stated, is not the case in their method. The data in Table I show that the extinction is proportional to the amount of glucose present. These data also illustrate the reproducibility of the method. The precision of the method is such that over 90 per cent of individual analyses of a known sample fall within 0.2  $\gamma$  of the expected result.

## SUMMARY

A very sensitive colorimetric determination of glucose based upon the reduction of ferricyanide ions is described. The range of the method is 1 to 9  $\gamma$  of glucose in 1 to 3 ml. of sample.

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# MECHANISM OF THE PHOSPHOGLYCERIC MUTASE REACTION

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Meyerhof and Kiessling (1) described the reaction, 3-phosphoglyceric acid  $\rightleftharpoons$  2-phosphoglyceric acid, which is catalyzed by phosphoglyceric mutase<sup>1</sup> and represents one of the steps of the glycolytic cycle. It is shown in this paper that 2,3-diphosphoglyceric acid, the ester isolated by Greenwald (2) from erythrocytes, acts as coenzyme in this reaction. The mechanism of the reaction was investigated with 3-phosphoglyceric acid labeled with P<sup>32</sup>; an exchange occurred between the monophosphate esters and the diphosphate ester.

In the experimental section use is made of the combined mutase and enolase reactions, 3-phosphoglyceric  $\rightleftharpoons$  2-phosphoglyceric  $\rightleftharpoons$  2-phosphopyruvic acid. At equilibrium at 20° the approximate concentration of the three reactants is 60, 10, and 30 per cent, respectively, according to Meyerhof and Kiessling (1), giving a ratio of 3-P-glyceric to 2-P-glyceric acid of about 6. From the data of Warburg and Christian (3) for the enolase equilibrium and the over-all equilibrium, 2-P-pyruvic/3-P-glyceric + 2-P-glyceric acid (4), one would calculate a value of only 3 for the above ratio.

It should now be possible to resolve this discrepancy by a direct determination of the mutase equilibrium with purified enzyme preparations in the presence of the coenzyme, 2,3-diphosphoglyceric acid. Previous attempts to purify mutase and to separate it from enolase (1, 5) were not successful, because of an apparent instability of the mutase to various fractionation procedures. This difficulty has not been encountered in the present work, and some preliminary fractionation steps are described.

## *Preparations and Methods*

*Preparation of Enzymes*—The crude enzyme was prepared by extracting perfused and ground rabbit muscle with 1.5 and then 1 volume of water. The pooled extract, after passage through gauze and cotton, was dialyzed against cold running tap water for 24 hours. A protein fraction precipitated from the extract by 0.55 saturated ammonium sulfate and then

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<sup>1</sup> The original designation of this enzyme as phosphoglyceromutase is not sufficiently descriptive in view of the occurrence of two isomeric phosphoglycerols.

reprecipitated by 0.5 saturated ammonium sulfate was used in some experiments, while in others the protein fraction precipitating between 0.4 and 0.5 saturation with ammonium sulfate was used. These fractions were dialyzed against cold tap water before use.

The crude dialyzed extracts contained a phosphatase which attacked 2,3-diphosphoglyceric acid.<sup>2</sup> The ammonium sulfate fractions were almost free of this phosphatase and of enolase and they were also activated to a larger extent by 2,3-diphosphoglyceric acid than were the crude extracts.

Purified enolase was precipitated from the mother liquor of crystalline glyceraldehyde-P-dehydrogenase (6) by raising the ammonium sulfate saturation from 0.71 to 0.85.

*Preparation of Substrate*—Small amounts of D(−)-3-phosphoglyceric acid were prepared by incubation of triose phosphate with crystalline triose-P-dehydrogenase, diphosphopyridine nucleotide, and arsenate. Larger amounts (labeled with P<sup>32</sup>) were prepared from yeast according to the method of Neuberg and Lustig (7) after incubation of the yeast in the presence of radioactive inorganic phosphate. After several crystallizations, the optical rotation before and after addition of molybdate corresponded to that previously found for D(−)-3-phosphoglyceric acid (8). Racemic 2-phosphoglyceric acid and 3-phosphoglyceric acid were prepared by oxidation of the corresponding glycerophosphates according to the method of Kiessling (9).

The isolation of 2,3-diphosphoglyceric acid from blood was carried out according to the method of Greenwald (2) with certain modifications. The brucine salt (10) instead of the barium salt was prepared and recrystallized twice from water. After drying at 110° over P<sub>2</sub>O<sub>5</sub> *in vacuo* the brucine salt contained 2.59 per cent P (calculated for the dihydrate, 2.73 per cent). The brucine was removed by chloroform extraction from a solution made alkaline to phenolphthalein with NaOH. The  $[\alpha]_D$  was +4.2° for the sodium salt and remained unchanged after addition of molybdate.

*Measurements of Enzyme Activity*—The activity of the phosphoglyceric mutase was measured spectrophotometrically and polarimetrically.

With the crude dialyzed enzyme in dilute solution the mutase reaction was the limiting step in the over-all reaction, 3-phosphoglyceric acid → lactic acid, and hence it was possible to show the effect of addition of 2,3-diphosphoglyceric acid. The disappearance of reduced diphosphopyridine nucleotide was followed in the Beckman spectrophotometer at 340 mμ in a reaction mixture containing all the necessary components (K<sup>+</sup>, Mg<sup>++</sup>, adenylic acid, enzymes) for rapid conversion of the product of

<sup>2</sup> The per cent inorganic P (in terms of total P) formed during 4 hours of incubation at 37° at pH 5, 7.4, and 8.3 was 21, 17, and 11, respectively.

the mutase reaction, 2-phosphoglyceric acid, to lactic acid. Another test system was based on the reaction, 3-phosphoglyceric  $\rightleftharpoons$  2-phosphoglyceric  $\rightleftharpoons$  2-phosphopyruvic acid, with purified mutase and an excess of purified enolase. The formation or disappearance of phosphopyruvate was followed spectrophotometrically at 240  $m\mu$  (3).

Formation of D(-)-3-phosphoglyceric acid from racemic 2-phosphoglyceric acid in the mutase reaction could be followed directly by polarimetric measurements according to the method of Meyerhof and Schulz

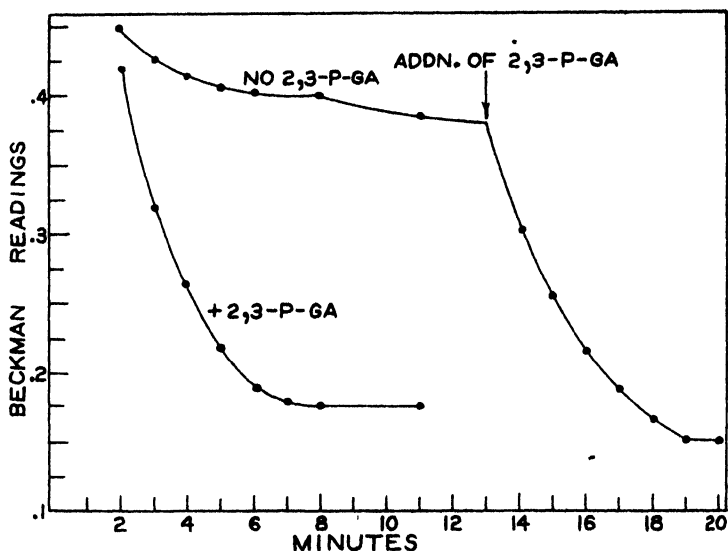


FIG. 1. Rate of conversion of D(-)-3-phosphoglyceric acid to lactic acid with and without catalytic amounts of 2,3-diphosphoglyceric acid (2,3-P-GA) ( $1 \times 10^{-5}$  M). The reaction mixture contained reduced diphosphopyridine nucleotide,  $Mg^{++}$ ,  $K^{+}$ , and adenylic acid and was incubated at pH 7.4 with dialyzed and diluted (1:10) muscle extract. The absorption of reduced diphosphopyridine nucleotide at 340  $m\mu$  was measured.

(8). A 1 dm. tube holding 1.3 ml. was used for the readings. Allowance was made for the (unnatural) L-2-phosphoglyceric acid remaining after the conversion of the (natural) D form.

### Results

**Spectrophotometric Measurements**—Fig. 1 shows the rate of conversion of natural 3-phosphoglyceric acid (prepared enzymatically from triose phosphate) to lactic acid in a dialyzed muscle extract diluted 10-fold. Addition of a catalytic amount of 2,3-diphosphoglyceric acid greatly

increased the rate of reaction. When 2,3-diphosphoglyceric acid was added alone (without 3-phosphoglyceric acid substrate), no reaction occurred. Other control procedures consisted in adding 2-phosphoglycerate, 2-phosphopyruvate, or pyruvate as substrates instead of 3-phosphoglycerate; with each of these the reaction was much more rapid than with 3-phosphoglyceric acid, showing that the over-all rate was limited by the mutase reaction. Iodoacetate ( $7 \times 10^{-4}$  M) did not inhibit the mutase reaction.

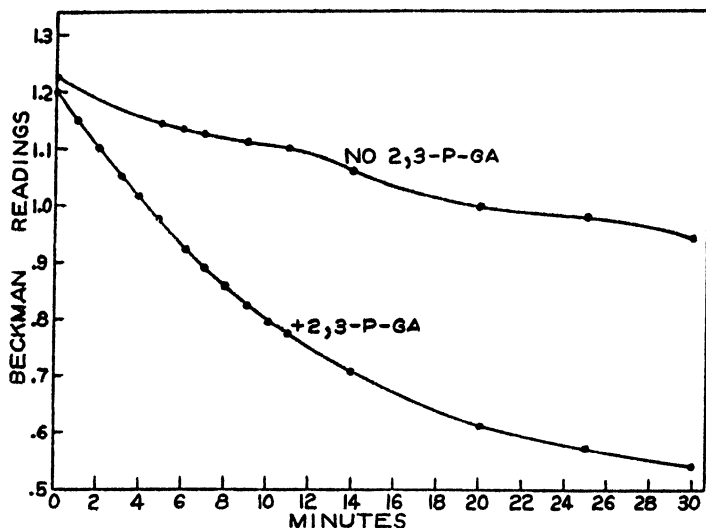


FIG. 2. Rate of disappearance of 2-phosphopyruvic acid with and without a catalytic amount of 2,3-diphosphoglyceric acid (2,3-P-GA) ( $3 \times 10^{-4}$  M). The substrate,  $2 \times 10^{-3}$  M racemic 2-phosphoglyceric acid, was first incubated at pH 7.4 with enough purified enolase so that equilibrium was established rapidly; then a mutase preparation obtained between 0.4 and 0.5 saturation with ammonium sulfate was added at zero time. The absorption of 2-phosphopyruvate at  $240 \mu$  was measured.

Fig. 2 shows the rate of disappearance of 2-phosphopyruvate as a function of mutase activity, enolase being present in excess. The experiment consisted in establishing first the enolase equilibrium by adding to a reaction mixture<sup>3</sup> containing 2-phosphoglyceric acid purified (mutase-free) enolase. The equilibrium was then displaced to the side of 3-phosphoglyceric acid by the addition of mutase purified by ammonium sulfate fractionation; here again the reaction was greatly accelerated in the pres-

<sup>3</sup> In this reaction mixture almost all of the cations were sodium and the addition of adenylic acid (which was necessary in the experiment in Fig. 1) was omitted. This was done in order to prevent a conversion of 2-phosphopyruvate to pyruvate, a reaction for which both potassium ions and adenylic acid are necessary.

ence of 2,3-diphosphoglyceric acid. With the same enzyme system the reverse reaction, the formation of 2-phosphopyruvate from 3-phosphoglycerate, was likewise accelerated by the addition of small amounts of 2,3-diphosphoglyceric acid.

*Polarimetric Measurements*—Some data obtained with the polarimetric method have been tabulated in a preliminary report (11); they show that  $6 \times 10^{-4}$  M 2,3-diphosphoglyceric acid gave a suboptimal and 4 times this concentration a maximal stimulation of mutase activity.

The polarimetric method (8) is based on the fact that in neutral solution, in the presence of molybdate ions, D-3-phosphoglyceric acid has a large negative rotation ( $[\alpha]_D = -745^\circ$ ), while L-2-phosphoglyceric acid (which is formed in equimolecular amounts during the reaction when racemic 2-phosphoglyceric acid is used as substrate) has a relatively small positive rotation ( $[\alpha]_D = +68^\circ$ ). An aliquot of 1.5 ml. was taken from the reaction mixture at different times and was precipitated with 0.25 ml. of 50 per cent trichloroacetic acid. After centrifugation, 1.0 ml. of the supernatant fluid was just neutralized to phenolphthalein with 0.4 to 0.5 ml. of NaOH, followed by the addition of 0.5 volume of 25 per cent ammonium molybdate. A time curve for the conversion of 2-phosphoglyceric acid to 3-phosphoglyceric acid and the catalytic effect of 2,3-diphosphoglyceric acid on this conversion is shown in Fig. 3.

#### *Phosphate Exchange Experiment*

The reaction mixture for the exchange experiment consisted of 7.0 ml. of a solution of the sodium salt of 2,3-diphosphoglyceric acid containing a total of 8.73 mg. of P, 2.3 ml. of D(-)-3-phosphoglyceric acid ( $P^{32}$ ) (prepared from yeast) containing a total of 3.08 mg. of P, and 3 ml. of enzyme solution (fraction obtained between 0.4 and 0.5 saturation with ammonium sulfate and dialyzed for 20 hours). The reaction mixture was incubated at pH 7.2 for 30 minutes at  $30^\circ$ . Protein was removed by heating at  $100^\circ$  for 10 minutes, followed by centrifugation. Neutral lead acetate (1.5 ml. of 25 per cent solution) was added to precipitate the mono- and diphosphoglyceric acids and the precipitate was decomposed with  $H_2S$ . The solution was neutralized to litmus by addition of brucine in methanol. The brucine salt of the diphosphate was crystallized five times from hot water to free it completely of the monophosphate and was converted to the sodium salt, which had  $[\alpha]_D = +5.0^\circ$  (unchanged by the addition of molybdate). The barium salt was precipitated by addition of barium acetate to the solution of the sodium salt and was dried at  $110^\circ$  over  $P_2O_5$  *in vacuo*. Analysis showed 10.39 per cent phosphorus and 55.57 per cent barium ( $C_4H_6O_{10}P_2Ba_2$ , calculated, P 10.25, Ba 56.82).

The first mother liquor of the brucine salt of 2,3-diphosphoglyceric acid was made alkaline with NaOH and extracted with  $CHCl_3$ . The

monophosphates were precipitated by addition of barium acetate and 2 volumes of ethanol. A separation of the barium salts of the 3- and 2-phosphoglyceric acids was achieved at a pH slightly acid to Congo red by

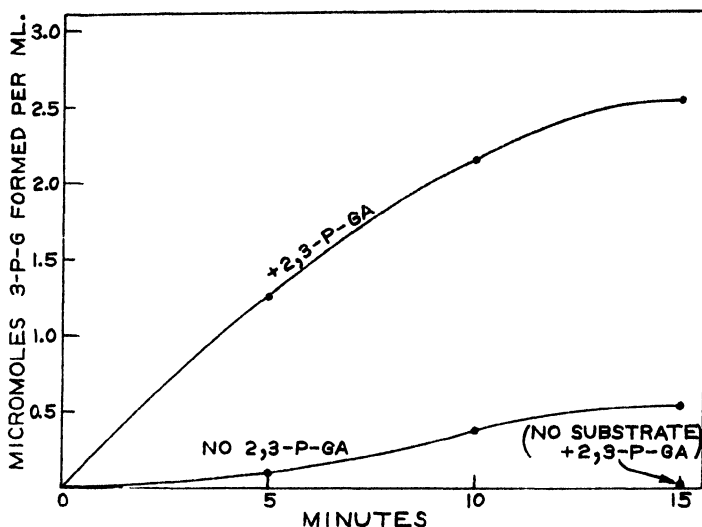


FIG. 3. Rate of conversion of 2-phosphoglyceric to 3-phosphoglyceric acid with and without a catalytic amount of 2,3-diphosphoglyceric acid (2,3-P-GA) ( $6 \times 10^{-4}$  M). The substrate,  $1.25 \times 10^{-2}$  M racemic 2-phosphoglyceric acid, was incubated at pH 7.4 with a mutase preparation obtained between 0.4 and 0.5 saturation with ammonium sulfate. The 3-phosphoglyceric acid formed was measured polarimetrically.

TABLE I

*Equilibration of Phosphoglyceric Acid ( $P^{32}$ ) with 2,3-Diphosphoglyceric Acid in Phosphoglyceric Mutase Reaction*

The reaction mixture contained purified enzyme, 3.1 mg. of P as  $\alpha$ (-)-3-phosphoglyceric acid ( $P^{32}$ ), and 8.7 mg. of P as 2,3-diphosphoglyceric acid.

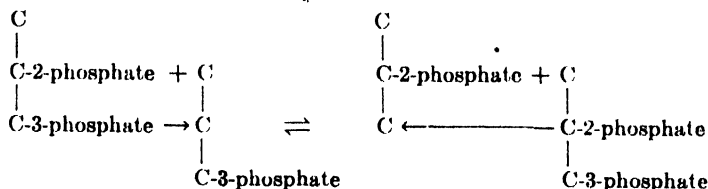
Sample	Initial	Final, 30 min.	
		Found	Calculated for 100 per cent equilibrium
	counts per min. per mg.	counts per min. per mg.	counts per min. per mg.
Monophosphate	29,200	12,430	7610
Diphosphate	0	4,860	7610

slow addition of 0.3 volume of ethanol. The barium salt of 3-phosphoglyceric acid was removed (plus a small amount of 2,3-diphosphoglyceric acid) by centrifugation and converted to the sodium salt. The change of rotation after addition of molybdate indicated that the solution contained

85 per cent of its total phosphorus as 3-phosphoglyceric acid; the remainder was 2,3-diphosphoglyceric acid and, since its specific radioactivity was known, a correction could be applied in the calculation of results.

Table I shows that during the 30 minute period of incubation the exchange of phosphate groups between the mono- and diphosphates had reached about 65 per cent of the value calculated for 100 per cent equilibration. It seems probable that the incubation period was too short for complete equilibration.

The mechanism of the phosphoglyceric mutase reaction may be formu-



lated in a manner analogous to that of the phosphoglucomutase reaction (12), as illustrated in the accompanying diagram.

#### SUMMARY

1. Phosphoglyceric mutase, the enzyme which catalyzes the reaction, 3-phosphoglyceric  $\rightleftharpoons$  2-phosphoglyceric acid, requires the presence of catalytic amounts of 2,3-diphosphoglyceric acid for its activity. This has been demonstrated in spectrophotometric and polarimetric tests for enzyme activity, with crude dialyzed muscle extract as well as with enzyme preparations purified by fractionation with ammonium sulfate.

2. The mode of participation of the diphosphate in the reaction has been investigated in an exchange experiment with  $P^{32}$  and the proposed mechanism of the reaction has been illustrated diagrammatically.

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# APPLICATION OF ELECTROPHORESIS-CONVECTION TO THE FRACTIONATION OF BOVINE $\gamma$ -GLOBULIN

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A method of fractionation of proteins in solution in which an electrophoretic adaptation of the principles of the Clusius column is utilized was suggested by Kirkwood in 1941 (1) and tested experimentally by Nielsen and Kirkwood (2) several years later. Recently an electrophoresis-convection apparatus of improved design has been described and successfully used in the fractionation of the pseudoglobulin of horse diphtheria antitoxin (3) and bovine serum proteins (4).

Fractionation occurs in a narrow vertical channel between two semi-permeable membranes, connecting an upper and lower reservoir. Separation depends upon the superposition of differential horizontal electrophoretic transport of the components on vertical convective transport of the solution as a whole. The vertical convective transport is controlled by the horizontal density gradient produced by the electrophoretic migration of the proteins across the channel. The result of the superposition of horizontal electrophoretic transport and vertical convective transport is movement of the mobile components from the top reservoir to the bottom reservoir at rates depending on their mobilities, with a relative enrichment of the top reservoir with respect to the slow components and the bottom reservoir with respect to the fast components.

The separation of a protein mixture possessing discrete mobility and isoelectric point spectra, *e.g.* serum, into its constituent proteins is accomplished by successive immobilization of the components at their respective isoelectric points and transport of the mobile components from the top reservoir of the apparatus.

In the case of a protein which migrates as a single boundary in an electric field but possesses a specified mobility distribution as revealed by reversible electrophoretic boundary spreading, *e.g.*  $\gamma$ -globulin, fractionation is accomplished by means of a modified isoelectric procedure in which the fractionation is carried out at a pH displaced by an arbitrary amount from the mean isoelectric point of the heterogeneous protein. Transport in the apparatus leads to a redistribution of the protein ions, such that the fractions withdrawn from the top and bottom reservoirs possess mobility dis-

\* Contribution No. 1293.

tribution differing from that of the original protein. Fractions possessing different mean mobilities and isoelectric points are obtained by proper choice of the pH. Transport proceeds to a stationary state, in which the top fraction is isoelectric at the operating pH.

Recently the authors (5) reported the results of two stages of fractionation of bovine  $\gamma$ -globulin prepared by ethanol precipitation, Fraction II of bovine plasma. By the modified isoelectric procedure described above,  $\gamma$ -globulin was separated into four fractions of different mean mobilities and isoelectric points. The  $\gamma$ -globulin was fractionated in Stage I at pH 6.7, several tenths of a pH unit removed from its mean isoelectric point. The top and bottom fractions resulting from this stage are designated as Fraction A and Fraction B, respectively. Fraction B served as the starting material for Stage II, which was also carried out at pH 6.7. The top and bottom fractions of this stage are designated as Fraction C and Fraction D,

TABLE I  
*Electrophoretic Properties of  $\gamma$ -Globulin Fractions*

Fraction	$-10^3 \times \bar{u}^*$	Isoelectric point†	$10^3 \times \beta$	$-10^3 \times \frac{\Delta \bar{u}}{\Delta \text{pH}}$
Original $\gamma$ -globulin	1.73	$\sim 6.5$	$0.67 \ddagger$	
A.	1.35	7.03	0.65	0.74
B	1.98-2.12			
C	1.63	6.47	0.67	1.3
D . . . . .	2.20	6.01	0.65	0.78

\* In barbital buffer, pH 8.7, and ionic strength 0.1.

† In cacodylate buffer (0.08 N sodium chloride-0.02 N sodium cacodylate).

‡ Non-Gaussian mobility distribution.

respectively. The electrophoretic properties of these fractions are summarized in Table I, where  $\bar{u}$  is the mean mobility ( $\text{cm}^2 \text{sec}^{-1} \text{volt}^{-1}$ ) at pH 8.7,  $\beta$  the standard deviation of the mobility distribution in the neighborhood of the isoelectric point, and  $\Delta \bar{u} / \Delta \text{pH}$  the slope of the mobility-pH curve in the neighborhood of the isoelectric point. The fractionation of  $\gamma$ -globulin has now been carried through two more stages, Stages III and IV, and four additional fractions obtained.

#### EXPERIMENTAL

**Material**—The bovine  $\gamma$ -globulin, Fraction II of bovine plasma, used in this investigation was kindly supplied by the Armour Laboratories, Armour and Company, Chicago.

**Electrophoretic Analysis**—The moving boundary technique of Tiselius (6) as modified by Longworth (7) was used in the electrophoretic analysis. Mobilities were determined by electrophoresis of a 1 per cent protein solu-

tion in barbital buffer, pH 8.7, and ionic strength 0.1, at a field strength of about 8 volts per cm. for 2 hours. Mobilities were calculated in accordance with the suggestions of Longworth and MacInnes (8). Isoelectric points were determined in cacodylate buffer (0.08 N sodium chloride-0.02 N sodium cacodylate).

Experiments in boundary spreading were carried out on 0.5 per cent solutions of the  $\gamma$ -globulin fractions equilibrated against cacodylate buffer. They were performed at the average isoelectric points of the proteins. The power consumption in these experiments did not exceed 0.015 watt per ml. Curves of the refractive index gradient were recorded photographically on Eastman Kodak Company (CTC) plates by the cylindrical lens schlieren technique. A diagonal knife-edge brought in from below the optical axis was used in the optical system.

It is a result of the theory of reversible boundary spreading, to be published elsewhere, that the mobility distribution of an inhomogeneous protein,  $q(u)$ , can be expressed in terms of the moments of the refractive index gradient curve taken about the centroidal axis by means of an infinite series, Equation 1.  $D$  is the diffusion constant, and  $E$  the electric field strength.

$$q(u) = \frac{1}{\sqrt{2\pi}\beta} e^{-u^2/2\beta^2} \left\{ 1 + \sum_{j=3}^{\infty} \frac{C_j}{j!} (-i)^j \alpha^j H_j(iu\sigma/Et_E\beta^2\alpha) \right\} \quad (1)$$

$$\beta^2 = (\sigma^2 - \sigma_0^2 - 2Dt_E)/E^2t_E^2$$

$$\sqrt{1 - 2(\sigma/\beta Et_E)}$$

$\sigma_0$  and  $\sigma^2$  are the second moments of the gradient curves at the moment of application and at time  $t_E$  after application of the electric field.  $\beta$  is the standard deviation of the mobility distribution.  $H_j$  is the  $j$ th Hermite polynomial. The coefficients  $C_j$  are related to the higher moments,  $\bar{x}^n$ , of the gradient curve, *e.g.*,

$$C_3 = \bar{x}^3/\sigma^3, C_4 = (\bar{x}^4/\sigma^4) - 3$$

If the gradient curves are Gaussian in form, *i.e.* the mobility distribution is Gaussian,  $\beta$  is identical to the heterogeneity constant  $h$  of Albery (9). Deviations from a Gaussian distribution of mobilities are given by the third and higher moments of the gradient curves. For proteins possessing either Gaussian or non-Gaussian mobility distribution,  $\beta$  may be calculated from Equation 2.  $D^*$  is the apparent diffusion constant calculated from the second moments of the gradient curves during electrophoresis. A plot of

$$D^* = (\sigma^2 - \sigma_0^2)/2t_E = D + (E^2\beta^2/2)t_E \quad (2)$$

$D^*$  versus  $t_E$  is a straight line which extrapolates back to the normal diffusion constant at zero time.  $\beta$  may be calculated from the slope  $\beta^2 E^2/2$ . The standard deviation of the mobility distributions are tabulated as  $\beta$ . The distribution may be assumed to be Gaussian unless otherwise specified.

*Fractionations*—The details of construction and operation of the electrophoresis-convection apparatus employed in this investigation have previously been described. Metal screws in the apparatus originally described have been replaced by plastic screws. Paper base bakelite 10-32 screws have been used with some success. However, it has been found that screws made with du Pont nylon, FM-10001, are more satisfactory. A photograph of one of the fractionation units is shown in Fig. 1. The

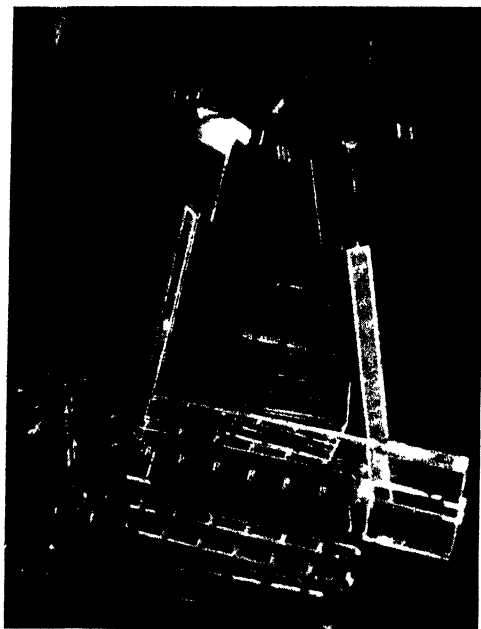


FIG. 1. Electrophoresis-convection apparatus

fractionation cell consists of a narrow vertical channel connecting upper and lower reservoirs.<sup>1</sup> The channel is formed by the space between two

<sup>1</sup> The volumes of the top and bottom reservoirs of the apparatus used in this investigation were 100 and 50 ml., respectively. Recently a small fractionation cell, Fig. 1, with top and bottom reservoir capacities of 15 and 10 ml., respectively, has been constructed and successfully used to separate  $\gamma$ -globulin from rabbit serum. The channel wall separation and the length of the channel in this apparatus are the same as in the one described previously. The width of the channel and the platinum electrodes are one-third that specified previously; the longitudinal ribs of the face plates have been eliminated. Except for these changes the dimensions of the cell are essentially the same as in the larger apparatus. In this apparatus, the 6-32 brass machine screws used to bolt the face plates to the cell block have been replaced by 10-32 paper base bakelite screws, thus eliminating the necessity for electrically insulating the heads and tips of the screws as well as the distortion of the electric field across the channel by the metal screws.

sheets of semipermeable membrane. The cell containing a solution of the protein to be fractionated is immersed in buffer solution and a homogeneous electric field applied across the channel by means of external platinum electrodes. Electrolysis products are prevented from reaching the membranes by circulation of the external buffer solution. Fractionations are carried out in a constant temperature cold room at 4°.

$\gamma$ -Globulin fractions withdrawn from the top and bottom reservoirs at the conclusion of the runs were filtered, dialyzed first against distilled water and then dilute saline solution at 4° to remove buffer salts, and finally lyophilized. The dried protein preparations dissolved readily to form clear and stable solutions at their isoelectric points. In a few cases it was necessary to filter off a small amount of suspended material. Solutions of the various fractions were equilibrated against barbital or cacodylate buffer and analyzed electrophoretically.

### Results

A composite of Fraction A and  $\gamma$ -globulin served as the starting material for Stage III.<sup>2</sup> This  $\gamma$ -globulin, which had a mobility of  $-1.51 \times 10^{-5}$  and an isoelectric point of 6.75, was from a different batch from that used in Stage I. Stage III was carried out in phosphate buffer, pH 7.6, which was about 0.6 pH unit on the alkaline side of the mean isoelectric point of the starting material. The fractionation was carried out in duplicate. The pertinent data are presented in Table II.

The electrophoretic properties of the resulting top cut, Fraction E, are quite striking. The mobility of Fraction E was  $-1.24 \times 10^{-5}$ . The mean isoelectric point was found to be 7.31, about 0.8 pH unit greater than that of the original  $\gamma$ -globulin ( $-1.73 \times 10^{-5}$ ). Furthermore, the heterogeneity constant was significantly lower than the values found for other top fractions, and  $\Delta\bar{u}/\Delta\text{pH}$  was considerably lower than the values for the other fractions. The material withdrawn from the bottom reservoir in Stage III is designated Fraction F. Fraction F, obtained in Run 1, had a mobility of  $-1.78 \times 10^{-5}$  and an isoelectric point of 6.51. The mobility of Fraction F obtained in Run 2 was  $-1.69 \times 10^{-5}$ .

The top and bottom fractions of Run 2 were recombined in proportion to their relative concentrations in the starting material. The mobility and heterogeneity constant of this composite were  $-1.45 \times 10^{-5}$  and  $0.59 \times 10^{-5}$ , respectively, which are to be compared with the calculated values of  $-1.54 \times 10^{-5}$  and  $0.67 \times 10^{-5}$ .

A composite of Fraction B and the  $\gamma$ -globulin possessing a mobility of  $-1.51 \times 10^{-5}$  appeared to be roughly comparable to the  $\gamma$ -globulin of

<sup>2</sup> Since the starting material for Stage III is a composite of Fraction A and unfractionated  $\gamma$ -globulin, it does not represent a true stage in the fractionation scheme. However, for convenience, we will refer to it as such.

mobility  $-1.73 \times 10^{-5}$ . This composite was fractionated under the same conditions as in Stage I. The resulting bottom fraction had a mobility of  $-1.91 \times 10^{-5}$ , an isoelectric point of 6.29, and a heterogeneity constant of  $0.75 \times 10^{-5}$ . This material possessed a Gaussian distribution of mobilities at its mean isoelectric point, although at pH 8.7 the electrophoretic pattern was non-Gaussian and skewed. Differences in the dependence of mobility upon pH for the various components of the fractions undoubtedly are responsible for this departure of the mobility distribution from a Gaussian

TABLE II  
*Fractionation of Bovine  $\gamma$ -Globulin*  
*Experimental Conditions*

age No.	Run No	pH	<i>E</i>	<i>t</i>	<i>C</i>
			<i>volts per cm.</i>	<i>hrs.</i>	<i>gm. per 100 ml.</i>
III	1	7.60	1.7	52½	2.8
	2	7.58	1.7	53½	2.4
IV		5.48	1.6	51½	2.3

*Properties of  $\gamma$ -Globulin Fractions*

Stage No.	Run No.	Fraction	Yield	$-10^5 \times \alpha^*$	Isoelectric point†	$10^5 \times \beta$	$-10^5 \times \frac{\Delta \alpha}{\Delta \text{pH}}$
			<i>gm. protein</i>				
III	1	Top (E)	1.1	1.24	7.31		0.35
		Bottom (F)	2.0	1.78	6.51	0.59	0.89
	2	Top (E)	1.2	1.25		0.55	
		Bottom (F)	1.5	1.69			
IV		Top (G)	0.6	2.25	5.74	0.63‡	0.64
		Bottom (H)	2.3	1.81	6.41	0.77‡	0.81

\* In barbital buffer, pH 8.7, and ionic strength 0.1.

† In cacodylate buffer (0.08 *N* sodium chloride-0.02 *N* sodium cacodylate).

‡ Non-Gaussian mobility distribution.

one. This fraction, which appeared to be the same as Fraction B, served as the starting material for Stage IV.

The operating pH in Stage IV was 5.5, or 0.8 pH unit on the acid side of the mean isoelectric point of Fraction B. The material withdrawn from the top reservoir, Fraction G, had about the same mobility as Fraction D. However, its isoelectric point was about 0.3 pH unit more acid than that of Fraction D, and its mobility distribution was non-Gaussian. The bottom cut, Fraction H, which had a mobility of  $-1.81 \times 10^{-5}$  and an isoelectric

point of 6.41, possessed a skewed non-Gaussian distribution of mobilities with a rather large standard deviation. The calculated mobility and heterogeneity constant for a composite of the two fractions, in proportion to their relative concentrations in the starting material, are  $-1.91 \times 10^{-5}$  and  $0.76 \times 10^{-5}$ , respectively. These calculated values are in excellent agreement with the experimental values quoted above for Fraction B.

0.25

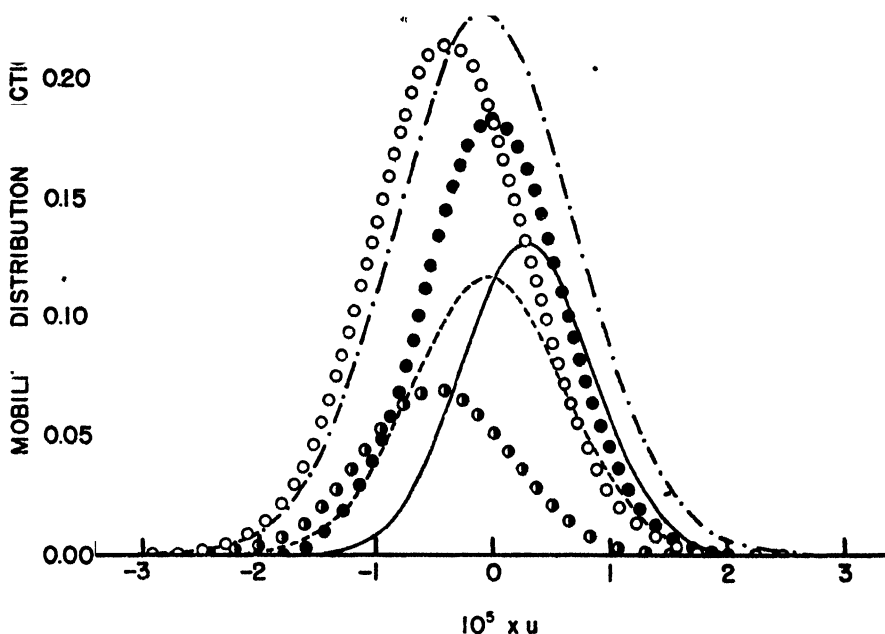


FIG. 2. Mobility distribution functions at pH 6.5 of the six unique fractions of bovine  $\gamma$ -globulin; --, Fraction C; O, Fraction D; —, Fraction E; ●, Fraction F; ●, Fraction G; - · -, Fraction H.

#### DISCUSSION

Bovine  $\gamma$ -globulin has been separated into eight fractions which constitute mean mobility and mean isoelectric point spectra, ranging from  $-1.25 \times 10^{-5}$  to  $-2.25 \times 10^{-5}$  and from 7.31 to 5.74 respectively. The various fractions can be distinguished from one another by at least two electrophoretic properties. Thus, Fractions C, F, and H have about the same mean isoelectric points but different mean mobilities at pH 8.7 and different mobility distributions at their respective mean isoelectric points. The considerable variation in  $\Delta\bar{u}/\Delta\text{pH}$  among the fractions is probably a reflection



of chemical and structural differences. The mobility distribution<sup>3</sup> of each of the six unique fractions at pH 6.5 has been normalized to an area corresponding to its weight fraction<sup>4</sup> of  $\gamma$ -globulin,  $-1.73 \times 10^{-5}$ , and is plotted in Fig. 2. Fractions G and H possessed non-Gaussian mobility distributions; however, as a first approximation, the mobility distribution can be adequately represented as a Gaussian probability function whose standard deviation is taken as that of the actual mobility distribution.

The theory of transport in an electrophoresis-convection channel predicts that the fractionation of a protein possessing a Gaussian mobility distribution, with specified first and second moments, will result in a top fraction which also possesses a Gaussian mobility distribution, having the same second moment as that of the original protein. The first moment will, of course, be different from that of the original protein. The theory also predicts that transport in the apparatus will proceed to a stationary state when the first moment of the mobility distribution of the material in the top reservoir vanishes. Both of these predictions have been approximately realized in these experiments.<sup>5</sup> The mobility distribution of the material

<sup>3</sup> It is assumed that, within the range of concentration and pH considered, the standard deviation of the mobility distributions of the  $\gamma$ -globulin fractions is independent of protein concentration and pH. It is also assumed that the slopes of the mobility-pH curves of all the protein ions present in a given fraction are the same and equal to the  $\Delta\bar{u}/\Delta pH$  of the fraction

<sup>4</sup> The relative concentrations of Fractions E and F were calculated on the assumption that the material fractionated in Stage III was Fraction A and not a composite of Fraction A and  $\gamma$ -globulin of mobility  $1.51 \times 10^{-5}$ .

<sup>5</sup> For purposes of characterization, the mean isoelectric points and standard deviations of the mobility distributions of the bovine  $\gamma$ -globulin fractions were determined in chloride-cacodylate buffer. This buffer was used because of the desirability of carrying out electrophoretic experiments in buffers containing

Fraction	Isoelectric point		$-10^5 \times (\Delta\bar{u}/\Delta pH)$		$10^5 \times \beta$	
	Chloride-cacodylate	Phosphate	Chloride-cacodylate	Phosphate	Chloride-cacodylate	Phosphate
Unfractionated $\gamma$ -globulin ( $-1.51 \times 10^{-5}$ )	6.75	6.33	0.75	0.97	0.61	0.66
A	7.03	6.19	0.74	1.15		
D	6.01	5.73	0.78	1.10		
E	7.31	6.44	0.35	0.92		
G	5.74	5.52	0.64	1.18		

univalent anions. In addition to the electrophoretic characterization of the fractions in chloride-cacodylate buffer, it also appeared desirable to determine isoelectric points and standard deviations of mobility distribution in phosphate buffer, since the fractionations were carried out in this buffer. Since preparation of this manuscript such determinations have been made on several fractions. For purposes of

fractionated in Stage IV was non-Gaussian and skewed at pH values removed from the mean isoelectric point. As a result, the top fraction obtained in this stage exhibited a non-Gaussian mobility distribution at its mean isoelectric point.

Although the bottom fractions from an original Gaussian distribution are not themselves Gaussian, they are not highly skewed. Therefore, we will represent them as Gaussian. The precision with which the moments of the curves of the refractive index gradient can be determined does not justify a more refined treatment.

The results of the fractionation of bovine  $\gamma$ -globulin illustrate the ease with which a heterogeneous protein can be fractionated by electrophoresis-convection. The large quantities of material that can be fractionated in a relatively short time, the ease of the manipulations, and the reproducibility of the fractionations promise to make electrophoresis-convection a valuable method for the subfractionation of the plasma fractions obtained by ethanol precipitation.

#### SUMMARY

Bovine  $\gamma$ -globulin has been separated into eight fractions which constitute mean mobility and mean isoelectric point spectra ranging from  $-1.25 \times 10^{-5}$  to  $-2.25 \times 10^{-5}$  at pH 8.7 and from 7.31 to 5.74 respectively.

This investigation demonstrates the applicability of electrophoresis-convection to the subfractionation of the plasma fractions obtained by ethanol precipitation.

comparison the resulting data are presented, along with the corresponding data obtained in chloride-cacodylate buffer, in the tabulation. It will be noted that there are rather large differences between the mean isoelectric points in chloride-cacodylate and in phosphate buffer. Although unfractionated  $\gamma$ -globulin has appreciably different isoelectric points in chloride-cacodylate and phosphate buffer, the standard deviations of the mobility distribution at the mean isoelectric point are approximately the same in the two buffers. Although the theoretical isoelectric condition of the top fraction at the operating pH was realized in Stage IV of the fractionation, the data obtained in phosphate buffer reveal that in the case of Stage I and Stage III the fairly good agreement between the theoretical isoelectric condition of the top fraction at the operating pH and the observed isoelectric condition based on measurements in chloride-cacodylate buffer is fortuitous. Obviously, this does not reflect upon the use of chloride-cacodylate buffer for purposes of electrophoretic characterization of the fractions. Failure to realize the isoelectric condition of the top fraction at the operating pH in Stages I and III may have been due to insufficient time of operation for attainment of a stationary state. However, this seems improbable. Theoretically, departure from the isoelectric condition is to be expected if some of the components of the heterogeneous protein produce different density increments per unit weight or possess different diffusion constants. A detailed treatment of this problem cannot be made at this time.

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# SERINE AND THREONINE DEAMINASES OF *ESCHERICHIA COLI*: ACTIVATORS FOR A CELL-FREE ENZYME\*

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Serine has been reported by many workers to be deaminated by a variety of bacterial cells and tissues, but this process was first studied in detail by Gale and Stephenson in 1938 (1). These workers followed the serine deaminase of *Escherichia coli* by measuring the release of ammonia by resting cell suspensions. The reaction proceeded anaerobically, thereby distinguishing it from the oxidative deaminases. Aging of cell suspensions caused a loss of deaminase activity, which could be prevented by the addition of reducing agents, such as glutathione or formate, or by adenylic acid. Chargaff and Sprinson (2, 3) studied serine and threonine deaminases, using toluene-treated suspensions of *E. coli* and found that pyruvate and  $\alpha$ -ketobutyrate, respectively, accumulated as the products of anaerobic deamination. Neither the *O*-ethers of serine nor phosphoserine were deaminated anaerobically. On the basis of these findings, Chargaff and Sprinson suggested desaturation as the mechanism. Binkley (4) obtained cell-free extracts of serine deaminase from *E. coli* which were inactivated by dialysis. The activity was restored by the addition of zinc ions. Lichstein *et al.* (5, 6) in studying the metabolic rôle of biotin inactivated the serine and threonine deaminases of *E. coli* by aging cell suspensions in phosphate buffer at pH 4. The activity was restored by the addition of biotin or adenylic acid. With a cell-free preparation, only yeast extract caused partial reactivation. From this evidence it was suggested that a coenzyme form of biotin is present in yeast extract (7).

In the present study, active serine and threonine deaminases have been obtained by growing *E. coli* in deep medium without carbohydrate. Vacuum-dried cells were prepared which contained most of the deaminase activity present in the living cells, and which differed from the living cells only in the requirement of adenylic acid for activation. The deaminases were freed from the cells by autolysis, and purified by ammonium sulfate precipitation and adsorption on calcium phosphate gel. The purified enzyme required both adenylic acid (AMP) and glutathione (GSH) for activity. The enzyme, as prepared, deaminated both serine and threonine. During serine deamination, simultaneous inactivation toward both substrates occurred.

\* This work was supported in part by the Office of Naval Research.

### Methods

**Culture**—The Crookes strain of *E. coli*, employed previously for studies of the arginine and glutamic acid decarboxylases (8) and of tryptophanase (9), was used. For active deaminase production, the cells were grown without aeration in a medium composed of 2 per cent tryptone, 1 per cent yeast extract, and 0.5 per cent dipotassium phosphate. To obtain a large crop of cells, 10 liter batches were grown in 2½ gallon reagent bottles. The medium was inoculated with 3 per cent of a 6 to 9 hour culture and incubated 12 to 14 hours at 37° (final pH 6.8 to 7.2). The cells were harvested with a Sharples centrifuge, the cell paste resuspended in 0.1 M phosphate buffer, pH 7.8, containing  $3 \times 10^{-3}$  M glutathione, and dried *in vacuo* over Drierite (yield, 3.5 gm. of dry cells per 10 liters of medium). The deaminase activity of the dried cells was approximately 560  $\mu$ l. of pyruvate per mg. of dry weight per hour with L-serine and about 890  $\mu$ l. of  $\alpha$ -ketobutyrate with L-threonine.

**Determination of Serine and Threonine Deaminase**—The deamination of serine and threonine has been shown to yield equimolar amounts of ammonia and pyruvate or  $\alpha$ -ketobutyrate (3). Since the vacuum-dried cells did not metabolize these keto acids, the deaminase activity could be followed by measuring the rate of keto acid formation. The enzyme activity was assayed in a 1 ml. volume containing the following: 0.1 ml. of 1 M phosphate buffer, pH 7.8, 0.1 ml. of  $7 \times 10^{-3}$  M adenylic acid, water to 0.89 ml., 0.01 ml. of enzyme and 0.1 ml. (1 mg.) of L-serine or L-threonine. Before addition of the enzyme and substrate, the assay tubes were brought to 37°. After substrate addition, the reaction was allowed to proceed for 10 minutes, then stopped with 0.5 ml. of 20 per cent trichloroacetic acid, the protein removed by centrifugation, and a 1 ml. portion of the supernatant removed for analysis.

A unit of serine or threonine deaminase was arbitrarily defined as the amount of enzyme necessary to form 1  $\mu$ M of pyruvate or  $\alpha$ -ketobutyrate in 10 minutes under the above experimental conditions.

**Pyruvate**—For most determinations, the direct method of Friedemann and Haugen (10) was used. Analyses by the extraction procedure of Friedemann and Haugen agreed with the results of the direct method.

**$\alpha$ -Ketobutyrate**— $\alpha$ -Ketobutyrate was also determined by the direct method of Friedemann and Haugen. The color was compared with a standard curve prepared from crystalline  $\alpha$ -ketobutyrate-2,4-dinitrophenylhydrazone, m.p. 204–205° (uncorrected). The color, when read in the Evelyn colorimeter with a 515 m $\mu$  filter, was linear up to 70  $\gamma$  of  $\alpha$ -ketobutyrate.

### Results

The enzyme assay was standardized by using graded amounts of dried cells. As is shown in Fig. 1, DL-threonine was deaminated more rapidly

than L-serine, the rate being proportional to cell concentration with each substrate. The formation of 20 to 120  $\gamma$  of pyruvate or 35 to 230  $\gamma$  of  $\alpha$ -ketobutyrate thus corresponds to 50 to 300  $\gamma$  of dry weight of cells. Since oxygen did not affect enzyme activity, assays were run aerobically. L-Threonine was used in later experiments with the purified enzyme and found to be deaminated more rapidly than the DL mixture, thus indicating possible inhibition by the D isomer.

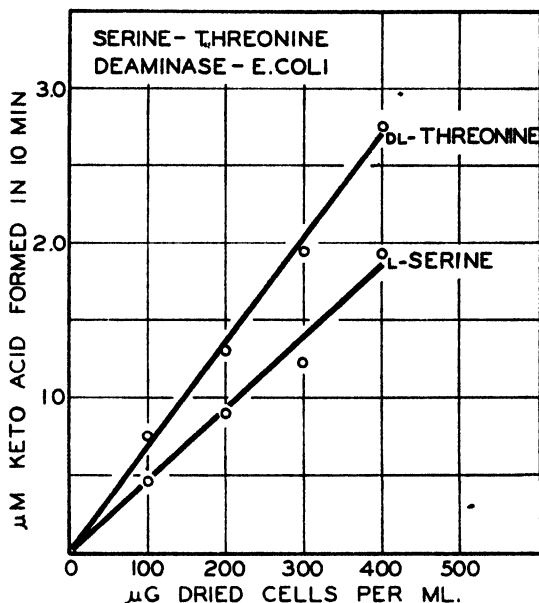


FIG. 1. Serine and threonine deaminase activity of vacuum-dried cells. Conditions, 0.1 ml. of M phosphate buffer, pH 7.8; 0.1 ml. (2.5 mg.) of adenosine-5-phosphate; 0.6 ml. of water; allowed to stand 5 minutes at 37°; cells as indicated and water to 0.9 ml.; 0.1 ml. (1 mg.) of L-serine or (2 mg.) DL-threonine; incubate 10 minutes at 37°; add 0.5 ml. of 20 per cent trichloroacetic acid. We wish to thank Dr. H. E. Carter for kindly furnishing the L-serine.

In the dried preparations, both deaminases required adenylic acid for activity, in contrast to the resting cells. In order to study the rôle of adenylic acid in the absence of interfering reactions, extraction of the enzymes and partial purification were undertaken.

#### *Cell-Free Enzyme*

4 gm. of vacuum-dried cells were suspended in 200 ml. of glutathione-phosphate buffer and the deaminases extracted by freezing, thawing, and autolysis. The cell-free extracts, obtained by centrifugation of the autolysate, contained about 50 per cent of the total activity. The enzymes

were precipitated by 40 per cent saturation with ammonium sulfate, adsorbed on calcium phosphate gel, and eluted with phosphate buffer. The details of the purification are shown in the flow sheet. The first eluate

*Flow Sheet for Purification of Serine and Threonine Deaminases*

Suspend 1 gm. vacuum-dried cells (4100 units serine deaminase, 4570 units threonine deaminase) in 50 ml. 0.1 M phosphate buffer, pH 7.8, containing  $6 \times 10^{-3}$  M GSH. Freeze and thaw twice, autolyze 4 to 5 hrs. at 37°; centrifuge

Discard	1870 units serine deaminase, 2420 units threonine deaminase Add $(\text{NH}_4)_2\text{SO}_4$ to 40% saturation; centrifuge
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Redissolve in 5 ml. 0.1 M phosphate buffer, pH 7.8, containing $3 \times 10^{-3}$ M adenylic acid; centrifuge	Discard
---	---------

Discard	1450 units serine deaminase, 1020 units threonine deaminase Add 50 ml. 1:10 dilution of $\text{Ca}_3(\text{PO}_4)_2$ gel, stir, centrifuge
---------	---

Wash with four 12 ml. portions distilled water	Discard
--	---------

Elute with 5 ml. M phosphate buffer, pH 7.8	Discard washings
---	------------------

593 units serine deaminase, 525 units threonine deaminase; 10 to 15% recovery  
Add adenylic acid to  $3 \times 10^{-3}$  M

from the calcium phosphate gel contained 10 to 15 per cent of the activity present in the dried cells. When stored in the frozen state, the enzymes were stable for several months; however, when stored at 0° without adenylic acid, inactivation occurred. The enzyme units recovered at each step

in the purification and the degree of resolution with respect to adenylic acid (and glutathione) are shown in Table I. The degree of resolution was obtained by expressing the increase in rate of deamination due to addition of the activator as per cent of the maximum rate (activator present). The relative serine and threonine deaminase activity at each step in the purification is shown in the last column of Table I. The purity index was expressed as the ratio of threonine deaminase units to protein content, as determined by the biuret test of Robinson and Hogden (11).

### *Characteristics of Serine and Threonine Deaminases*

The purified extract contained both serine and threonine deaminases in virtually the same proportions as were present in dried cells (Table I). This suggested a similarity of properties, if not the identity of the enzymes.

TABLE I  
*Purification of Serine and Threonine Deaminases*

Step No	Activity		Resolution		L-Serine DL-Threo- nine
			AMP	GSH	
	units	per cent	per cent	per cent	
1. Cell suspension (in GSH-0.1 M phos- phate, pH 7.8)	4100	100	82	32	0.90
2. Cell-free enzyme (from cells frozen, autolyzed at 37°, 5 hrs.)	1870	46	-		0.77
3. Ppt. from 40% saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1450	35			1.42
4. Eluate from Ca <sub>2</sub> (PO <sub>4</sub> ) <sub>2</sub> gel (in 1 M phosphate, pH 7.8)	593	15	94	99	1.13

For comparative purposes, the enzyme characteristics were investigated by use of both serine and threonine as substrates.

The influence of serine and threonine concentration upon enzyme activity is shown in Fig. 2. The half maximum activity was obtained with approximately 305  $\gamma$  per ml. of L-serine or L-threonine. This corresponds to a Michaelis constant (12) for substrate-enzyme of  $3.5 \times 10^{-3}$  and  $3.0 \times 10^{-3}$  mole per liter, respectively. The presence of the D isomer of serine or threonine depressed deamination by about 50 per cent. Cysteine, which differs structurally from serine only in the polar group on the  $\beta$ -carbon, has been shown by Desnuelle and Fromageot (13) to be deaminated by *E. coli* in a manner similar to serine, the products being pyruvate, ammonia, and hydrogen sulfide. In the light of these similarities, desulfurase activity as determined by pyruvate formation was measured, but no activity was found. Binkley (4) has suggested that enolase, in addition to converting



2-phosphoglyceric acid to phosphopyruvate, also catalyzes the deamination of cysteine and serine with the formation of pyruvate.

**Adenylic Acid**—As with the dried cells, both serine and threonine deaminases of the purified extract were activated by adenylic acid. Adenosine-5-phosphate obtained by hydrolysis of adenosine triphosphate (ATP) or prepared by the yeast fermentation of adenosine<sup>1</sup> gave the same activation (Table II). Other nucleosides and nucleotides including adenosine, adenosine-3-phosphate, and ATP were ineffective, thus indicating that adenosine-5-phosphate is specifically required. As is shown in Table II, yeast

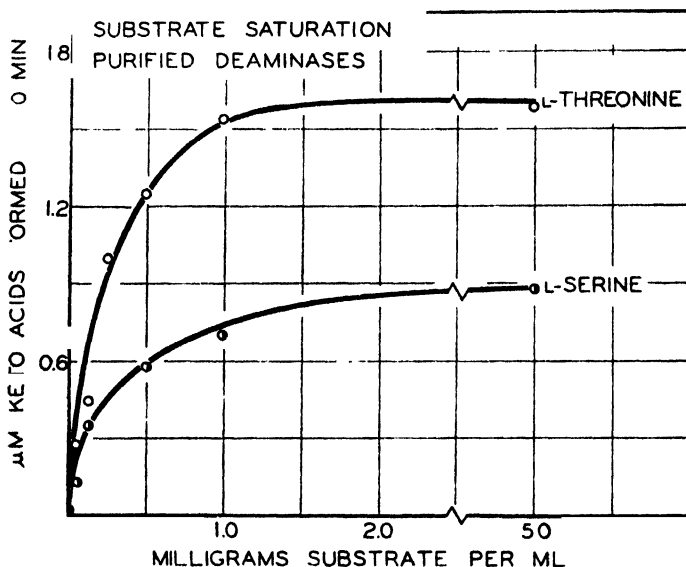


FIG. 2. Substrate saturation curves for serine and threonine deaminases. Conditions as in Fig. 1 except as follows: 0.1 ml. of  $1.3 \times 10^{-4}$  M glutathione added with adenosine-5-phosphate; allowed to stand 10 minutes at 37° with enzyme; substrate levels as indicated.

extract, which was found by Lichstein (7) to activate these deaminases in the absence of adenylic acid or biotin, was ineffective with the purified enzymes.

The adenylic acid activation curves for both deaminases are shown in Fig. 3. The concentration necessary for half maximum activation is about 400  $\gamma$  per ml. with serine and about 245  $\gamma$  per ml. with threonine. These correspond to Michaelis constants of  $1 \times 10^{-3}$  and  $0.7 \times 10^{-3}$  mole per liter respectively.

<sup>1</sup>We wish to thank the Ernst Bischoff Company, Ivoryton, Connecticut, for a supply of adenosine-5-phosphate.

**Glutathione**—For activity, the purified deaminase required glutathione in addition to adenylic acid (Table III). Reducing agents, including

TABLE II  
*Activation of Serine and Threonine Deaminases by Adenosine-5-Phosphate*

Additions	Keto acid formed	
	Pyruvate	$\alpha$ -Ketobutyrate
None	0	0
Adenosine	0	0
Adenosine-3-phosphate	0	0
Adenosine-5-phosphate (from ATP)	53	120
Adenosine-5-phosphate (yeast fermentation)	49	123
Adenosine triphosphate.	3.3	4.3
Guanylic acid	0	0
Yeast extract (1 mg. per ml.)	0	0

Additions  $7 \times 10^{-4}$  M except as indicated.

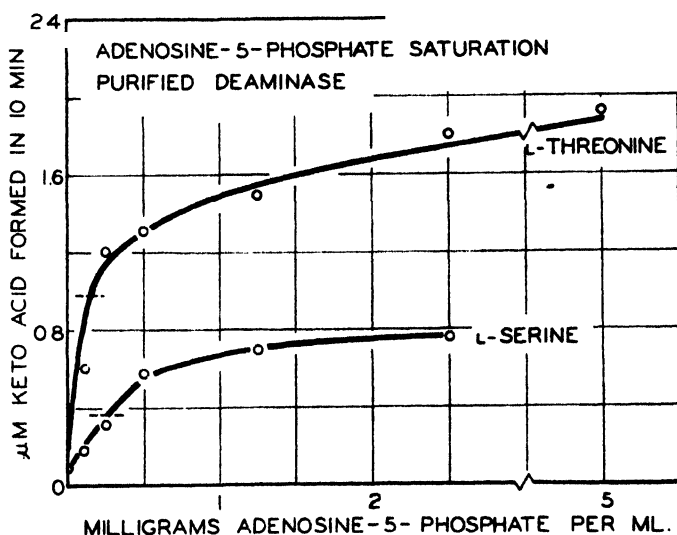


FIG. 3. Adenosine-5-phosphate saturation curve for serine and threonine deaminases. Conditions as in Fig. 2 except 0.1 ml. (1 mg.) of L-threonine and adenylic acid levels as indicated. We are indebted to Dr. E. E. Howe of Merck and Company, Inc., Rahway, New Jersey, for a sample of L-threonine.

cysteine, sodium thioglycolate, and ascorbic acid, were ineffective. However, sodium sulfide and sodium cyanide did cause partial reactivation. Since glutathione forms complexes with heavy metals and acts as a reduc-

ing agent as well, other complex-forming agents were tested. Bipyridyl, 8-hydroxyquinoline, histidine, pyrophosphate, and gum arabic were with-

TABLE III

*Activation of Partially Purified Serine and Threonine Deaminases*

Conditions as in Fig. 3 except that glutathione and adenylic acid were added as indicated.

Additions	Concentration $M \times 10^{-3}$	Keto acid formed	
		Pyruvate	$\alpha$ -Ketobutyrate
		$\gamma$	$\gamma$
None		0.5	0.0
AMP	6.8	2.3	3.6
GSH	12.8	6.1	4.9
GSH	6.8, 12.8	107	134

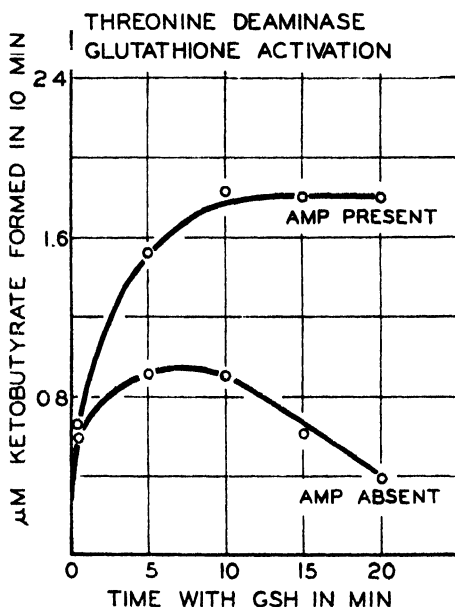


FIG. 4. Activation of threonine deaminase by glutathione. Conditions as in Fig. 1 except as follows: Enzyme incubated with glutathione for the times indicated; adenylic acid added before or after incubation with glutathione as indicated; substrate, DL-threonine (2 mg. per ml.).

out effect, thereby suggesting the presence of oxidized functional sulfhydryl groups rather than heavy metal inhibition. This possibility was

further indicated by the fact that the enzyme is inhibited by  $10^{-5}$  M mercuric, silver, and cupric ions, and is not reactivated by glutathione.

Enzyme activation by glutathione as a function of time is shown in Fig. 4. When glutathione was incubated with the enzyme in the presence of adenylic acid, activation was complete in 10 minutes. However, in the absence of adenylic acid partial activation occurred, followed by inactivation.

As with the dried cells, deaminase activity was proportional to the enzyme concentration. However, the rate of threonine deamination was

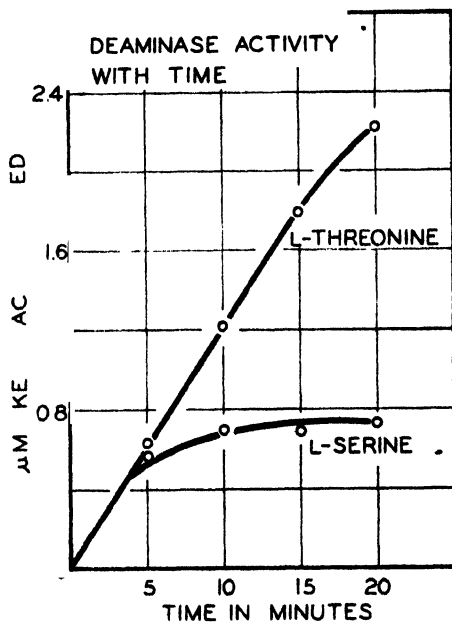


FIG. 5. Serine and threonine deaminase activity as a function of incubation time. Conditions as in Fig. 2 except for the time of incubation with substrate as indicated.

linear with time, whereas the rate of serine deamination decreased rapidly, approaching zero between 5 and 15 minutes (Fig. 5). The inactivation of the enzyme by serine occurred only during the reaction, that is, incubation of the enzyme in the presence of serine, but in the absence of the activators, or in the presence of the end-products, was without effect. Furthermore, increasing the concentration of each reactant did not preserve the rate of deamination. Similarly, the addition of yeast extract or a preparation of *E. coli*, as a source of cofactors, neither preserved nor reactivated serine deaminase. The mechanism of the enzyme inactivation is not known.

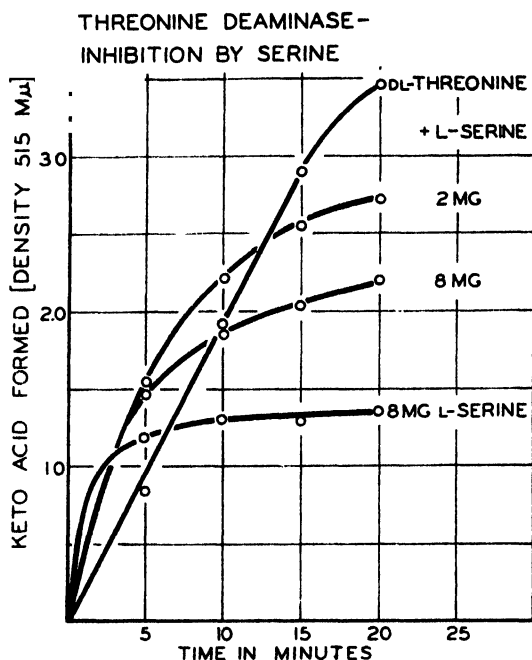


FIG. 6. Deaminase activity on mixtures of serine and threonine. Conditions as in Fig. 2 except for substrate mixtures and incubation times as indicated.

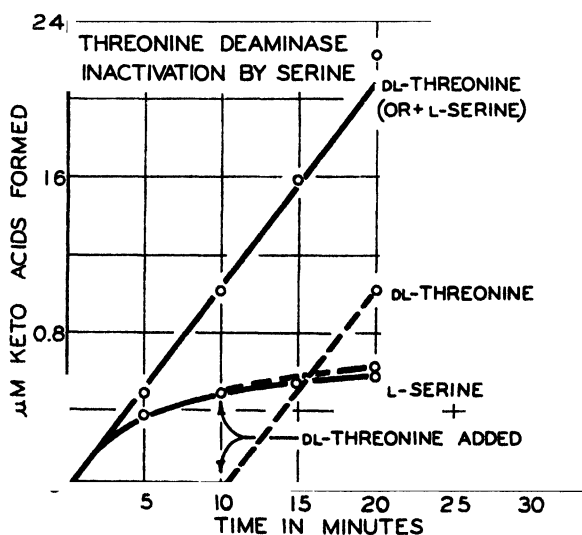


FIG. 7. Inactivation of threonine deaminase by serine. Conditions as in Fig. 2 except for substrate additions and incubation times as indicated.

Deamination of serine and threonine by the purified extract may be due to the presence of two similar enzymes or one enzyme catalyzing the two reactions. As is shown in Table I, the purified preparation contained the deaminases in about the same proportion as the dried cells; also, as shown in Table III, both deaminases were activated by adenylic acid and glutathione. Attempts were therefore made to show the presence of separate enzymes catalyzing the two deaminations. The rate of keto acid formation in the presence of threonine with increasing levels of serine is shown in Fig. 6. The data indicate that the initial rate of deamination of a serine-threonine mixture was intermediate between the rates obtained with serine or threonine alone. Since the rates were not additive, competition for a single enzyme is suggested. A second fact which suggests the identity of the enzymes is the loss of deaminase activity for both substrates during incubation with the mixture, the rate being proportional to the serine concentration.

To show the presence of an independent threonine deaminase, conditions were employed in which serine deaminase was inactive; *i.e.*, after 10 minutes incubation of the enzyme with L-serine (Fig. 5). The results, as recorded in Fig. 7, show that after incubation with serine the enzyme did not deaminate threonine, thereby indicating that threonine deaminase was incapable of functioning independently of serine deaminase. This suggests the identity of the two enzymes. Competitive inhibition of threonine deamination by serine appeared unlikely, since deamination of a serine-threonine mixture occurred at approximately the same rate as with threonine alone (Fig. 7).

#### SUMMARY

Serine and threonine deaminases have been obtained from *Escherichia coli* and partially purified.

The enzyme has been resolved and shown to require adenosine-5-phosphate and glutathione for activity.

Serine and threonine deaminases occurred in the extracts in approximately the same ratio as the dried cells, were activated by the same concentrations of adenylic acid and glutathione, and threonine deamination disappeared when serine deaminase was inactivated. These facts suggest that both substrates may be activated by a single enzyme.

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# THE METABOLISM OF URIC ACID IN THE NORMAL AND GOUTY HUMAN STUDIED WITH THE AID OF ISOTOPIC URIC ACID\*

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The present report deals with an investigation of uric acid metabolism in man. The procedure has been to inject intravenously a single dose of isotopic uric acid, as its lithium salt, into normal and gouty human subjects maintained in nitrogen balance on a calorically adequate low purine diet.

During the experimental period uric acid was repeatedly isolated from serial urine samples, and, from the isotope concentration found in the products isolated, the magnitude of the pool of miscible uric acid in the body, as well as the turnover rate of this pool, was calculated.

In establishing the protocol for such an experiment it was obviously desirable to keep the necessary dose of injected isotopic uric acid as small as feasible. By so doing one reduces to a minimum the insult of the procedure, and the change in uric acid metabolism which may be presumed to follow the injection of uric acid. With stable isotopes it is rarely possible to keep the dose of test substance as low as can be achieved with radioactive isotopes, in view of the smaller dilution tolerated by the analytical instrument. In the present instance, as will be seen, we have been able to keep the dose relatively low in comparison with the quantity of uric acid in the bodies of the subjects.

The isotopic uric acid employed was therefore prepared from the most enriched source of  $N^{15}$  available to us and by a procedure which would result in the introduction of isotope into two of the four possible positions. The resultant product had an isotope concentration half as high as that present in the initial isotope source.

## EXPERIMENTAL

Isotopic uric acid labeled with  $N^{15}$  in positions 1 and 3 has been prepared by two methods. In both cases the first product was isotopic urea formed by the ammonolysis of molten diphenyl carbonate (1) in the presence of

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copper catalyst (2). The ammonia was prepared from ammonium nitrate  $N^{15}H_4NO_3$  (Eastman Kodak Company) which was stated to contain 62 atom per cent excess of  $N^{15}$ . In the first synthesis isotopic urea was converted to barbituric acid (3), nitrated (4), and the 5-nitrobarbituric acid reduced with tin and hydrochloric acid to uramil (5). In the second preparation advantage was taken of the innovation introduced by Cavalieri, Blair, and Brown (6) in which acetamidomalonic ester<sup>1</sup> was condensed with urea to yield acetyluramil which, on hydrolysis, gave the desired uramil. In both syntheses, the isotopic uramil was condensed with potassium cyanate (7), and the potassium pseudourate cyclized to uric acid by the procedure of Fischer (8). Purification was effected by precipitation with acetic acid from lithium carbonate solution (9).

Elementary analyses were performed upon the product of a non-isotopic trial run.

**Analysis**— $C_5H_4O_2N_4$ . Calculated. C 35.7, H 2.4, N 33.3  
Found. " 35.7, " 2.4, " 33.4

Solutions of isotopic uric acid were prepared for intravenous injection by dissolving the required amount of uric acid in 10 ml. of a sterile 5 per cent aqueous glucose solution which contained an appropriate amount of lithium carbonate. The solution was warmed to 60°, cooled, and then 20,000 units of crystalline penicillin G were added. The clear solution was allowed to stand at room temperature for 8 hours and was made up to a final volume of 200 ml. by the addition of 5 per cent glucose solution. Such solutions were injected intravenously over a period of 30 minutes and no untoward effects were observed in any case.

The total urine output of each subject was collected in 12 hour portions, and each sample was analyzed for uric acid by the method of Kern and Stransky (10) as modified by Archibald,<sup>2</sup> adapted to urine (11). From 94 to 98 per cent of the chromogenic material in urine determined by this method was found to be destroyed by uricase. Pending isolation of urinary uric acid, the samples were preserved by freezing.

Each urinary uric acid sample that was isolated was dated as of the mid-point of the time interval during which the corresponding urine sample was voided. Isolations were performed upon pooled samples collected over periods of 5 to 12 hours during the 5 to 8 days succeeding injection of isotopic uric acid. A typical 500 ml. urine sample was treated with 150 ml. of 10 per cent nickelous acetate (12) solution, and 20 per cent sodium carbonate solution was then added until a pH of about 11 was achieved.

<sup>1</sup> A sample of acetamidomalonic ester was received as a gift from Winthrop-Stearns, Inc., to whom the authors are deeply indebted.

<sup>2</sup> R. M. Archibald, personal communication.

The precipitate was collected by centrifugation and stirred with 50 ml. of 6 N hydrochloric acid. The insoluble residue, crude uric acid, was twice washed with water by centrifugation and was further purified by solution in 2.5 N sodium hydroxide and precipitation by the addition of concentrated hydrochloric acid. The purification was repeated and this was followed by a final precipitation from aqueous lithium carbonate solution by addition of acetic acid (9). Small amounts of pigment which accompanied the uric acid in certain cases were readily removed by treatment of the alkaline solution with norit.

*Analysis*— $C_5H_4O_6N_4$ . Calculated. C 35.7, H 2.4, N 33.3  
Found. " 35.5, " 2.5, " 33.1

Ammonia (13) and urea (14) were isolated for isotope analysis from random urine samples during each experiment.

Creatinine (15) and total nitrogen (16) determined on each 24 hour urine sample proved to be quite constant, suggesting adequate sampling and the maintenance of a positive nitrogen balance throughout the experimental period.

Both the normal and the gouty subjects remained in the medical wards of the Peter Bent Brigham Hospital during the experimental period. They were maintained in slightly positive N balance on constant diets calculated (17) to contain purines equivalent to 2.4 to 3.1 mg. of uric acid per day. It was found that a preliminary period of at least 3 days on this diet was required to achieve a reasonably constant urinary uric acid output. The same dietary regimen was maintained during the succeeding experimental period.

Determinations of the concentration of serum uric acid (11) revealed only small variations during the period of hospitalization. Analyses of serum samples collected immediately before and after injection of isotopic uric acid exhibited either no demonstrable change in concentration or at most a temporary rise of less than 10 per cent of the analytical value.

The first three subjects, D. C., R. B., and G. W., were all purportedly normal, young, white adult males, free from any clinical manifestations of gout. Two of these subjects had serum uric acid levels in the neighborhood of 6 mg. per cent (normal 3 to 6 mg. per cent) and one of the subjects, R. B., was found to have an asymptomatic subclinical icterus with a serum bilirubin concentration of 1.63 mg. per cent. Toward the middle of the experimental period (as indicated in Fig. 3), subject G. W. received anterior pituitary adrenal corticotropic hormone<sup>3</sup> equivalent to 60 mg. per

<sup>3</sup> Anterior pituitary adrenocorticotropin (batch H-1701) was kindly provided by Dr. John R. Mote, Armour Laboratories, Chicago, Illinois.

day of standard LA-1-A (Armour) in four divided doses for 2 successive days. No other medication was administered to any of the subjects.

Subject B. S., a white male of 36 years, had a history of two acute attacks of gout, the first 2 years, the second 2 weeks prior to the present experiment. No tophi have ever been noted. Subject A. L., a 63 year-old white male, had a long history of severe gout, going back possibly 25 years, with numerous acute attacks of gouty arthritis during the interval 1937-1940, followed by wide-spread articular involvement. Several tophi of considerable size had been surgically excised from his elbows, knees, and ankles and several tophi up to about 2 cm. in diameter still persisted. This subject represented a most severe case of tophaceous gout.

The isotope abundance of the nitrogen samples was determined with a 60° mass spectrometer.<sup>4</sup> Some preliminary isotope analyses were carried out at Yale University Medical School by Dr. Henry Hoberman, to whom the authors are indebted.

#### DISCUSSION

Let

$A$  = mg. uric acid in the organism capable of prompt mixing with intravenously injected uric acid

$a$  = mg. isotopic uric acid injected at zero time, a small quantity compared with  $A$

$I_i$  = atom per cent excess isotope in the uric acid injected

$I$  = atom per cent excess isotope in urinary uric acid at any time  $t$  (days) thereafter

$I_0$  = atom per cent excess isotope in the uric acid of the miscible pool immediately after injection and mixing

$\frac{dA}{dt}$  = the turnover rate, in mg. per day of the uric acid in this pool

Let it further be assumed that  $A$  is a constant, that  $dA/dt$  is likewise a constant, that the uric acid which enters the pool at all times after the injection is devoid of an excess of isotope, and that the urinary uric acid has at any time the same isotope concentration as the uric acid present at that time in the pool. A quantity  $K$  may now be defined as the fraction

$$(I) \quad K = \frac{dA/dt}{A}$$

of all the uric acid in the miscible pool which is replaced by non-isotopic uric acid each day.

At time  $t + dt$ , the quantity of isotope present in the pool is proportional to

$$(II) \quad A(I - dI) = AI - IdA$$

<sup>4</sup> Model M-60, Process and Instruments, 60 Greenpoint Avenue, Brooklyn, New York, built under the supervision of Dr. Joseph Greenspan and Mr. Kenneth Roach.

whence

$$(III) \quad A(dI/dt) = I(dA/dt)$$

or

$$(IV) \quad \frac{dA/dt}{A} = K = \frac{dI/dt}{I}$$

Integrating this expression between the limits  $t_0$  and  $t$ , we obtain

$$(V) \quad K = \frac{\ln I - \ln I_0}{t}$$

From this relationship it is apparent that, granting the foregoing assumptions, a plot of  $\ln I$  against  $t$  will give a straight line whose negative slope is  $K$  and whose intercept is  $\ln I_0$ . Accordingly, we have treated our isotope data in this fashion (Figs. 1 to 5) and have obtained the most probable values for the intercept and the slope, in each case, by the method of least squares. The fact that the experimental values are distributed close to straight lines, as expected from Equation V, tends to confirm the validity of the assumptions which we have made. From the computed value of  $I_0$ , *i.e.* the antiln of the intercept, the magnitude of the miscible pool of uric acid may readily be calculated.

$$(VI) \quad aI_t = I_0(a + A)$$

or

$$(VII)^* \quad = a \left( \frac{I_t}{I_0} - 1 \right)$$

If the quantity of uric acid in this miscible pool,  $A$ , is multiplied by  $K$ , the fraction of this pool which is replaced each day by non-isotopic uric acid, the product will equal  $dA/dt$ , the turnover of the pool in mg. per day (from Equation I), the quantity of uric acid in the pool that is replaced daily by non-isotopic uric acid. It may be pointed out that all the quantities discussed thus far are evaluated without consideration of any quantitative uric acid analyses. The results of these calculations are given in Table I.

Subject G. W. was given adrenocorticotropin (ACTH) intramuscularly in order to determine whether the rise in the urinary excretion of uric acid known to follow the increase in adrenal cortical hormone secretion (19, 11) is due to improved uric acid clearance or a more rapid production of uric acid. Unfortunately, this subject failed to show a significant rise in urinary uric acid excretion, although adequate adrenal cortical activation

\* See Rittenberg and Foster (18).

was suggested by a fall in circulating eosinophils, a rise in fasting blood sugar, and a doubling of urinary 17-ketosteroid excretion on the 2nd day of a 48 hour ACTH administration. The plot of the isotope data (Fig. 3) shows no significant deviation from the original slope during or after ACTH administration; so that it may be safely assumed that there was no dilution of the isotope by an increase in uric acid formation in this case. Owing to the lack of an excessive uric acid excretion, these observations do not determine the relative importance of improved renal clearance and uric acid production.

TABLE I  
*Magnitude of Miscible Pool of Uric Acid and Its Turnover*

Subject	$a$ = dose injected	$I_1$ = isotope injected	$I_0$ = antilog of intercept	$A$ = miscible pool	$K$ = - slope	$KA$ = turnover	$B$ = urinary excretion	$KA - B$ = surplus	$C$ = body weight	$A/0.7C$ = mean concentration in body water	Mean serum level
	mg.	atom per cent excess	atom per cent excess	mg.	day <sup>-1</sup>	mg. per day	mg. per day	mg. per day	kg.	mg. per cent	mg. per cent
D. C. Normal	59.9	29.7	1.27	1,341	0.533	715	602 ±12*	113	73	2.6	6.0 ±0.3*
R. B. "	56.3	29.7	1.36	1,173	0.591	693	563 ±13	130	62	2.7	6.2 ±0.1
G. W. "	75.0	29.6	1.82	1,145	0.757	867	616 ±24	251	76	2.2	4.4 ±0.2
B. S. Gouty	111.0	29.7	0.62	4,742	0.524	2485	468 ±31	2017	74	9.2	6.9 ±0.2
A. L. "	75.0	29.6	0.12	18,450	0.463	8530	416 ±8	8114	75	35.1	9.6 ±0.1

\* Average error.

Certain points may be mentioned concerning the three subjects, R. B., D. C., and G. W. (Figs. 1, 2, and 3). The quantity of uric acid in the miscible pool ranged, in these three normal men, from 1100 to 1300 mg., of which between 53 and 76 per cent was daily replaced. This would require the introduction into the pool of from 690 to 870 mg. of new uric acid daily. Yet despite the fact that during this period these subjects were in as nearly a balanced state as possible, the urinary excretion of uric acid was in each case significantly lower than this figure. It ranged from 560 to 620 mg. per day, leaving a surplus of uric acid of from 110 to 250 mg. per day.

The fact that the normal man apparently forms more uric acid each day than appears in his urine leaves open the question as to the fate of this

surplus. Possible losses of uric acid in the feces and sweat have not been explored in the present experiment. The likelihood that a portion of the uric acid undergoes degradation to smaller fragments is indicated by the presence of small, though significant, concentrations of isotope in many of

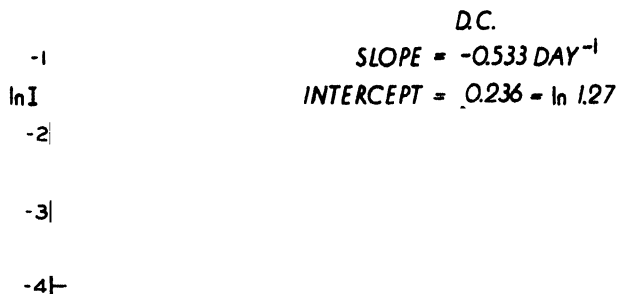


FIG. 1. Normal subject D. C. The natural logarithm of the isotope concentration has been plotted against time.

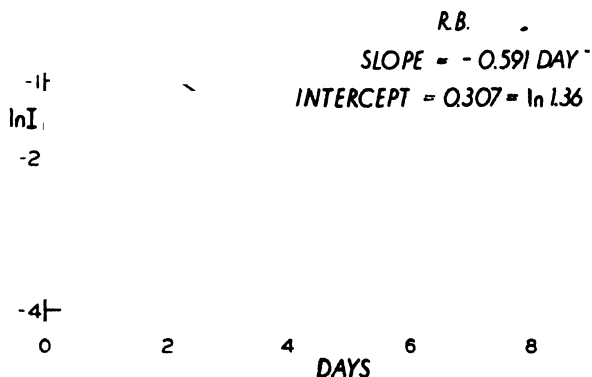


FIG. 2. Normal subject R. B.

the randomly collected samples of urinary urea and ammonia (see Table II). Because of the great dilution of isotope in these products, the precise evaluation of this fate of uric acid would require a much greater dose than was administered in the present experiments.

If the body water is taken as maximally equal to 70 per cent of the body weight, it is of interest to compute the mean concentration of uric acid

that would be achieved if all of the uric acid present in the miscible pool were uniformly distributed throughout all of the water of the body (*cf.* Table I, column " $A/0.7C$ "). The values obtained in these calculations were, in each of the normal subjects, approximately one-half of the mean concentration of uric acid in the corresponding sera, from which it is in-

$$\begin{array}{r} \text{G.W.} \\ \text{SLOPE} = -0.757 \text{ DAY}^{-1} \\ \text{INTERCEPT} = 0.600 = \ln 1.82 \\ -1 \\ \ln I \end{array}$$

↑ ↑ ↑ ↑  
A C T H

<sup>3</sup>  
DAYS

FIG. 3. Normal subject G. W. Adrenal corticotrophic hormone was injected at the times indicated by the arrows. (See the text.)

TABLE II  
*Concentration of  $N^{15}$  in Urinary Ammonia and Urea Samples*

Subject	Day	Ammonia	Urea	Corresponding uric acid
		<i>atom per cent excess</i>	<i>atom per cent excess</i>	<i>atom per cent excess</i>
D. C. Normal	1.5	0.011	0.007	0.536
	13.5	0.012		
R. B. "	1.5	0.007	0.023	0.580
	8.0	0.004		0.004
G. W. "	6.5	0.010	0.004	0.021
B. S. Gouty	1.0		0.005	0.376
	3.0	0.016		0.129
A. L. "	1.0	0.001	0.008	0.082

ferred either that some uric acid in certain portions of the body is excluded from the miscible pool or that the uric acid concentration is far from uniform throughout the body fluids and is much lower in some fluids than it is in serum.

Patient B. S. (Fig. 4), a man with a history of two attacks of acute gout,

without demonstrable tophi and free of apparent disease during the experimental period, was found to have nearly 4800 mg. of uric acid in his miscible pool, or about 4 times as much as did the normal subjects. 52 per cent of this was replaced daily, requiring the introduction into this pool of some 2500 mg. of non-isotopic uric acid each day. Since his mean daily uric acid excretion was less than 500 mg., it follows that some 2000 mg. of uric acid were being disposed of each day by routes other than urinary excretion. Computation of the hypothetical mean concentration of uric acid throughout all the body fluids in this case gave a value in excess of the mean serum uric acid concentration.

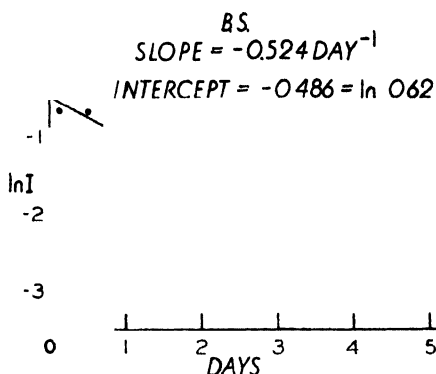


FIG. 4. Gouty subject B. S.

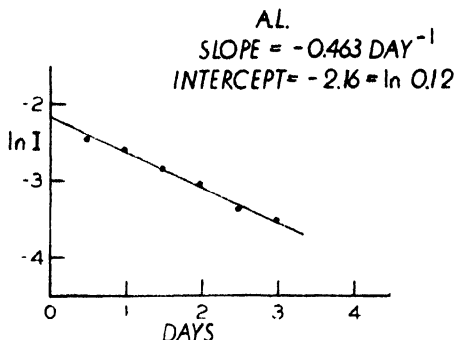


FIG. 5. Severely gouty subject A. L.

The severely gouty subject, A. L. (Fig. 5), exhibited changes in the same direction but to a much more marked degree. His miscible pool of about 18,500 mg. is approximately 15 times as large as that found in the normal subjects. Of this some 46 per cent, or about 8500 mg., was replaced by



non-isotopic uric acid each day, of which only 400 mg. could be accounted for as excreted in the urine, leaving approximately 8000 mg. of uric acid for which no accounting could be made. The uric acid present in his miscible pool, if uniformly distributed throughout the water of his body, would require a mean concentration of 35 mg. per cent, or about 4 times the concentration found in this patient's serum, an altogether improbable figure and far in excess of the expected solubility of uric acid. The magnitude of this pool, in the case of subject A. L., is such as to render improbable the assumption that miscible uric acid, in the gouty subject, is confined to uric acid in solution, and it has therefore been concluded that some of the urate known to be present in the solid phase as tophi has contributed to this pool.

Whereas there is no reason to doubt the approximate correctness of the assumptions initially made in so far as they apply to the normal subjects, an additional factor, the presence of urate in the solid phase, must be considered in explaining the results obtained with the patients suffering from gout. Such deposits were palpably present in subject A. L., and may well have occurred in subject B. S. also. That a portion of the material in the solid phase, presumably in the superficial layers, contributed to the rapidly miscible pool, has already been indicated, but it is noteworthy that the 18 gm. of uric acid estimated to be present in the miscible pool of uric acid in patient A. L. fell far short of clinical estimates of the mass of visible and palpable tophi.

The possibility must therefore be entertained of a large reservoir of initially non-isotopic urate present in the solid phase, which equilibrates *slowly* with the uric acid present in the *rapidly* miscible pool. Such a process would result in a gradual replacement of isotopic uric acid in the pool by non-isotopic uric acid and would be indistinguishable, in our type of experiment, from the dilution of isotope in the pool incident to the introduction of newly formed uric acid. The turnover values computed for the gouty patients may therefore be taken as representing the sums of these two processes and, in the absence of other evidence, the contribution of the individual processes cannot be evaluated. Experiments are currently under way, designed to study the occurrence of equilibration between plasma uric acid and tophi in the gouty individual.

#### SUMMARY

Isotopic ( $N^{15}$ ) uric acid has been administered in a single intravenous injection to three normal and two gouty subjects maintained on a low purine diet, and the isotope concentration of the urinary uric acid followed for a time thereafter.

Analysis of the results obtained from the normal subjects reveals that the

injected uric acid was promptly diluted by a miscible pool of uric acid containing about 1200 mg. The rate of formation of uric acid was calculated from the rate of fall of isotope concentration and was found to exceed the rate of urinary excretion of uric acid by 20 per cent or more. Evidence is presented for the view that a portion of uric acid undergoes catabolic breakdown in man.

In the gouty subjects studied the miscible pool of body uric acid was found to be up to 15 times larger than in the normal, so large as to require that a portion of the solid phase urates, joint deposits and tophi, be included in this pool.

The authors wish to acknowledge the assistance of Dr. Sidney Soloway and Mr. Frank J. Rennie in conducting the isotope analyses, and Mrs. Eleanor Schroeder in the preparation of samples. They are also indebted to Dr. G. W. Thorn for helpful criticism and constructive aid.

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# A MICROCOLORIMETRIC METHOD FOR THE DETERMINATION OF CITRIC ACID

## II. A NOTE ON THE SUBSTITUTION OF FERROUS SULFATE FOR HYDRAZINE SULFATE AS THE REDUCING AGENT\*

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In 1947 we (1) described a microcolorimetric method, with a range of sensitivity from 10 to 60  $\gamma$ , for the determination of citric acid in small samples of blood and urine. This method was based on the conversion of citric acid to pentabromoacetone and on the subsequent reaction between pentabromoacetone and alcoholic sodium iodide with the development of a yellow color complex.

Hydrazine sulfate was utilized at two stages in this procedure for the reduction of free bromine and manganese dioxide. This reagent, which was originally introduced by Goldberg and Bernheim (2), was very satisfactory for this purpose. Unfortunately, the prolonged use of this reducing agent led to the development of undesirable skin reactions in several of the laboratory workers. This prompted a search for an alternative reducing agent which might be free of this objectionable feature. A number of substances were tested on both blood and urine samples, two of which were most promising. Hydroxylamine proved satisfactory for the analysis of citric acid in urine, but gave results with blood plasma or serum which were about twice as high as those obtained with hydrazine. Arsenic trioxide in 18 per cent solution gave the same result as did hydrazine, and equally good recoveries of added citric acid. However, the toxicity of this reagent militated against its adoption.

We then explored the suitability of a procedure which had been recommended by Pucher, Sherman, and Vickery (3) for this purpose; *viz.*, the addition of ferrous sulfate in excess.

Goldberg and Bernheim (2), in the development of their method for the determination of citric acid, substituted hydrazine sulfate for ferrous sulfate to avoid certain difficulties encountered in the use of the latter reagent. They found it difficult to distinguish the end-point when ferrous sulfate was used, inasmuch as the color of the resulting ferric salt was indistinguish-

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able from that of free bromine. This objection has now been overcome by the use of a constant amount of a saturated solution of ferrous sulfate. This was determined experimentally to be about twice the amount required for complete reduction of the free bromine and manganese dioxide present under the analytical conditions of the method. During the next step of the procedure, which consists of the extraction of the pentabromoacetone with heptane, the iron sulfates which are insoluble in heptane remain behind in the aqueous phase.

Our microcolorimetric method has been modified in the following manner by substitution of ferrous sulfate for hydrazine sulfate as the reducing agent.

TABLE I  
*Comparison of Citric Acid Determination in Plasma with Hydrazine and Ferrous Sulfate*

Plasma No.	Citric acid		Difference
	Hydrazine	Ferrous sulfate	
	mg. per cent	mg. per cent	per cent
1	1.9	1.6	-15
2	2.1	2.0	-5
3	2.2	2.2	0
4	3.3	3.4	+3
5	1.8	1.6	-11
6	2.4	2.1	-12
7	3.4	3.3	-3
8	1.8	1.8	0
9	1.7	1.7	0
10	2.5	2.2	-12

*Ferrous Sulfate Reagent*—A saturated solution of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (about 40 gm. diluted to 100 cc. of solution with the addition of 1 cc. of approximately 2 N  $\text{H}_2\text{SO}_4$ ) is freshly prepared before use. It is shaken for 5 minutes to insure saturation.

*Preliminary Bromination*—Preliminary bromination is carried out as previously described up to the point at which excess free bromine is removed by reduction. After the mixture has reached room temperature, the bromine fumes are removed by suction. 2 cc. of the ferrous sulfate solution are added and the mixture is shaken. The procedure is then continued as previously described.

*Oxidation of Citric Acid to Pentabromoacetone*—2.5 cc. of  $\text{KMnO}_4$  are added instead of 2.0 cc., as originally described, as the larger amount results in a more rapid formation of the manganese suspension. After standing in the water bath at about  $18^\circ$  for 30 minutes, suction is again applied

to remove bromine fumes. 6 cc. of ferrous sulfate solution are added, and the mixture is shaken and allowed to stand for about 3 minutes. Precautions must be taken to insure the complete reduction of free bromine which may adhere to the sides of the stopper. Suction is again applied

TABLE II  
*Comparison of Citric Acid Determination in Urine with Hydrazine and Ferrous Sulfate*

Urine No.	Urine  cc.	Citric acid per 24 hrs.		Difference  per cent
		Hydrazine	Ferrous sulfate	
		mg.	mg.	
	0.1	468	417	-11
	0.6	454	426	-6
	0.6	423	390	-8
	0.6	316	297	-6
	1.0	254	233	-8
	1.0	243	222	-9
	1.0	260	242	-7
	1.0	226	201	-11

TABLE III  
*Recovery of Citric Acid Added to Plasma and Urine*

Sample No.	Citric acid added	Citric acid found	Citric acid recovered
	γ	γ	per cent
Plasma 1	0	17.8	
" 1	4.6	22.6	104
" 1	9.2	26.8	98
" 2	0	28.0	
" 2	23.7	51.3	98
Urine 1	0	20.3	
" 1	10	31.1	108
" 1	20	39.0	94
" 1	30	47.8	92

to remove any traces of bromine in the air space above the solution before the final extraction with heptane.

### Results

A comparison of the values obtained in urine and blood with hydrazine and ferrous sulfate as reducing agents is given in Tables I and II. In Table III the results are given of recovery experiments of citric acid added to plasma and urine, by using ferrous sulfate.

The recovery of added citric acid with ferrous sulfate is the same as that previously obtained with hydrazine. It will be noted, however, that, in both blood and urine, lower values are found with ferrous sulfate than with hydrazine. This would suggest that, of the two, ferrous sulfate is a more specific reducing agent for this reaction.

In our original description of the method in which hydrazine was used, we stated that it was possible to interrupt the analysis after the preliminary bromination, and prior to extraction of this reaction mixture with heptane.

With the substitution of ferrous sulfate, it has been found that an occasional plasma specimen, the analysis of which has been interrupted at this point and refrigerated overnight, will yield slightly higher citric acid values of the order of 0.2 to 0.5 mg. per cent. Hence, this procedure is not recommended for plasma, but it may be used with urine. If a plasma analysis cannot be completed up to the final heptane extract on the same day, it is recommended that the trichloroacetic acid filtrate be prepared and kept refrigerated.

I wish to express my appreciation to Dr. Ephraim Shorr for his constant advice and encouragement.

#### SUMMARY

In the microcolorimetric citric acid method which we have previously described, the continuous use of hydrazine sulfate as the reducing agent often led to undesirable skin reactions. A saturated solution of ferrous sulfate, as previously introduced by Pucher, Sherman, and Vickery, was found to be a satisfactory and innocuous substitute.

The appropriate conditions have been described for the use of ferrous sulfate as the reducing agent in this microcolorimetric method.

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# FURTHER STUDIES ON THE METABOLISM OF 3-HYDROXY-ANTHRANILIC ACID BY RAT LIVER SLICES AND HOMOGENATES

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Previous studies (1) demonstrated that 3-hydroxyanthranilate, an intermediate in the metabolism of tryptophan, was converted by liver slices to compounds possessing nicotinic acid activity for the test organism *Lactobacillus arabinosus* 17-5. The nicotinic acid analyses were made after acid treatment of the samples. These studies have been extended to determine the effect of the amount of tissue used, time of incubation, addition of certain enzyme inhibitors, and of anaerobic conditions on the apparent conversion of 3-hydroxyanthranilate to nicotinic acid. Different treatments of the incubation mixture, namely  $N H_2SO_4$ , glacial acetic acid, or  $N NaOH$  prior to nicotinic acid analysis, have demonstrated the complex nature of the end-products produced. The data indicate, however, that appreciable quantities of quinolinic acid are produced. These results are reported in this paper.

## EXPERIMENTAL

Unless indicated otherwise, liver tissue from young adult rats was used as the enzyme source. Liver slices and liver homogenates were prepared and incubated in Warburg vessels at  $37^\circ$  in the presence of the substrate and 3 ml. of Krebs-Ringer-phosphate buffer (pH 7.4). The 3-hydroxyanthranilic acid used was synthesized as described by Nyc and Mitchell (2). The nitro derivative was reduced according to the method of Ewins (3).<sup>1</sup> The recrystallized material was dissolved in buffer and 1 mg. was added per flask. When experiments were conducted with  $N_2$  as the gas phase, the substrate was added as a homogenized suspension in 0.3 ml. of buffer to the side arm and was tipped into the center compartment after flushing the system with  $N_2$ .

After incubation the content of the flasks was transferred to Erlenmeyer flasks and 10 ml. of  $N H_2SO_4$  added. The samples were autoclaved for 30 minutes, and after neutralization and dilution, the nicotinic acid activity was determined with *L. arabinosus* as the test organism. Control flasks

<sup>1</sup> We are indebted to Dr. L. M. Henderson, University of Illinois, for supplying some of the 3-hydroxyanthranilic acid used in these studies.



without added substrate were included in each series and the nicotinic acid contributed by the enzyme preparation determined. Corrections for these blank values, which approximated 40  $\gamma$  for 300 mg. of homogenate, were made in all cases. The substrate incubated without the enzyme preparation showed no nicotinic acid activity.

The possibility of quinolinic acid being a product of the enzyme system from rat liver was investigated, since quinolinic acid is excreted by rats fed tryptophan or 3-hydroxyanthranilic acid (4). Glacial acetic acid was shown to be more effective in decarboxylating quinolinic acid than treatment with 1 or 2 *N* acid. The incubation mixtures were made to a volume of 10 ml. and equal aliquots treated with *N* H<sub>2</sub>SO<sub>4</sub> or with glacial acetic acid. 5 ml. aliquots of the incubation mixture were heated to boiling to coagulate the proteins, filtered, and evaporated to dryness. Glacial acetic acid (0.5 ml.) was then added, the mixture autoclaved for 30 minutes, and the nicotinic acid produced determined as described above. Control tests were treated in the same manner.

The enzyme inhibitors studied were added to the main compartment of the Warburg vessels prior to the addition of the enzyme preparation. Control tests were conducted to show that these additions did not inhibit the growth of *L. arabinosus*. Detailed conditions applicable to the individual experiments are shown in Tables I to IV.

#### RESULTS AND DISCUSSION

Liver slices as compared to homogenates as the enzyme source were investigated. The tissues were homogenized in a Potter-Elvehjem homogenizer in Krebs-Ringer-phosphate buffer. It was found that liver homogenates were a good source of the enzyme system (Table I). The conversion of substrate to nicotinic acid by slices and homogenates ranged from 5.1 to 6.7 per cent, which is in good agreement with previous results obtained with liver slices (1). No conversion occurred with the use of boiled tissue preparations.

It was of interest to examine the test conditions with respect to the amount of tissue used and the time of incubation. In these tests comparative sample treatments after incubation (*N* H<sub>2</sub>SO<sub>4</sub> and glacial acetic acid) were used. As shown in Table II, the amount of nicotinic acid produced from the substrate increased in a linear fashion as the amount of tissue was increased to approximately 75 mg. of tissue per flask, as determined with both sample treatments.

Larger quantities of nicotinic acid were found, however, after glacial acetic acid treatment than after *N* H<sub>2</sub>SO<sub>4</sub> treatment, indicating that appreciable amounts of quinolinic acid were produced by this enzyme system

from 3-hydroxyanthranilate.<sup>2</sup> The ratios of the values obtained after acetic acid treatment as compared to H<sub>2</sub>SO<sub>4</sub> treatment were reasonably

TABLE I

*Relative Effectiveness of Liver Slices and Homogenates in Converting 3-Hydroxyanthranilate to Nicotinic Acid-Active Compounds*

1 mg. of 3-hydroxyanthranilate, 300 mg. of tissue; 3 hours incubation at 37°; treatment with N H<sub>2</sub>SO<sub>4</sub>.

Liver sample	Nicotinic acid produced	Per cent conversion, molar basis
	$\gamma$	
Slices	53.6	6.62
	54.4	6.73
	51.4	6.35
" (boiled)	2	0.25
	2	0.25
Homogenate	50.2	6.20
	41.3	5.08
	46.7	5.77

TABLE II

*Effect of Tissue Concentration and Sample Treatment on Amount of Nicotinic Acid Produced*

1 mg. of 3-hydroxyanthranilate per flask; incubation at 37° for 2 hours.

Homogenized liver per flask	Nicotinic acid produced		$\frac{(b)}{(a)}$
	N H <sub>2</sub> SO <sub>4</sub> treatment (a)	CH <sub>3</sub> COOH treatment (b)	
mg.	$\gamma$	$\gamma$	
10	7.1	40.2	5.6
25	13.4	87.1	6.5
50	27.7	163.3	5.9
75	42.3	278.0	6.6
100	45.7	340.7	7.5
150	46.5	312.0	6.7
200	38.4	269.4	7.0
300	43.6	336.0	7.7

constant, ranging from 5.6 when 10 mg. of tissue were used to 7.7 when 300 mg. were added per flask.

These results suggested that glacial acetic acid treatment gave a more

<sup>2</sup> Dr. L. M. Henderson (private communication) has also observed that appreciable quantities of quinolinic acid are produced from 3-hydroxyanthranilate by a similar enzyme system.

sensitive measurement of the quinolinic acid produced than did  $\text{H}_2\text{SO}_4$  treatment in that greater amounts of nicotinic acid were produced and that the relative values obtained with both treatments reflected the amount of tissue added. Other tests were conducted to determine how much free and bound nicotinic acid was formed from 3-hydroxyanthranilate by this system. The analysis of water extracts and extracts treated with  $\text{NaOH}$  (which releases bound nicotinic acid (or amide) but does not decarboxylate quinolinic acid) revealed that relatively small amounts of free or bound nicotinic acid were produced. The amounts found ( $2 \gamma$  or less) were within the error of measurement of nicotinic acid. It may be concluded, therefore, that quinolinic acid or other compounds capable of forming nicotinic acid derivatives active for *L. arabinosus* when treated with  $\text{N H}_2\text{SO}_4$  or, more effectively, when treated with glacial acetic acid but not by  $\text{N NaOH}$ , are the major products of this system.

It should be pointed out, however, that the production of small amounts of nicotinic acid by the enzyme system from 3-hydroxyanthranilate would be physiologically significant. As an alternate mechanism, the excess 3-hydroxyanthranilate may be metabolized by the animal to quinolinic acid, which is excreted. Since the end-products of this system are apparently very complex, the products of the reaction are referred to throughout this paper as nicotinic acid-active compounds or the equivalent, although it is recognized that they are not the major products of the system but are the compounds measured by *L. arabinosus*.

Other studies conducted on the effect of time of incubation revealed that maximal values were obtained in 30 to 60 minutes when determined with  $\text{H}_2\text{SO}_4$  treatment and within 5 minutes time with  $\text{CH}_3\text{COOH}$  treatment. Further, the values were lower in many cases when the incubation period was prolonged, as determined by  $\text{CH}_3\text{COOH}$  treatment. This is in contrast to the definite increases noted in the values obtained with  $\text{H}_2\text{SO}_4$  treatment after the 30 minute tests. For example, after incubating for 5 minutes the values obtained by  $\text{H}_2\text{SO}_4$  and  $\text{CH}_3\text{COOH}$  treatment were 12 and 185  $\gamma$ , respectively; after incubating for 30 minutes, 23 and 119  $\gamma$ , respectively. The basic data for these experiments are shown in Table III. These findings illustrate the differences in the nature of the end-products of the reaction present at different time intervals of incubation. This seems apparent, since, if quinolinic acid were the only product present in quantity at a given time, the relative values obtained by  $\text{H}_2\text{SO}_4$  and  $\text{CH}_3\text{COOH}$  treatment would reflect the quantities present. Further, the excess of homogenized liver present (300 mg.) may have contributed additional enzyme systems capable of metabolizing quinolinic acid to compounds released at different rates by the sample treatments used.

No significant inhibition in the conversion of 3-hydroxyanthranilate to nicotinic acid metabolites was observed when the following enzyme inhibitors were added: NaCN (0.001 M final concentration), sodium arsenite (0.01 M), dinitrophenol (0.0001 M), or NaF (0.02 M) with either sample treatment.<sup>3</sup> In one experiment the conversion was increased when cyanide was added and more strikingly when arsenite was added. The latter observation may be of significance in that arsenite has been shown to inhibit oxidative decarboxylations (5). This inhibition may have reduced the

TABLE III

*Effect of Time of Incubation and Sample Treatment on Apparent Conversion of 3-Hydroxyanthranilate to Nicotinic Acid*

1 mg. of 3-hydroxyanthranilate, 300 mg. of homogenized liver per flask.

Time of incubation	N H <sub>2</sub> SO <sub>4</sub> treatment	CH <sub>3</sub> COOH treatment
	$\gamma$	$\gamma$
30	53.5	
120	67.5	
5	12	185
15	20	125
30	23	119
60	26	155
120	22	99
5	25	221
30	44	165
5	27	216
30	40	195

amount of breakdown of quinolinic acid to other metabolic products inactive in the test system for nicotinic acid.

An extensive series of experiments was conducted with N<sub>2</sub> as the gas phase. In every instance a reduction in the amount of nicotinic acid produced was observed with N<sub>2</sub>, as determined by H<sub>2</sub>SO<sub>4</sub> treatment of the samples. This reduction ranged from 30 to 88 per cent in individual experiments and was observed both for liver slices and homogenates. The results obtained on aliquots of the same incubation mixtures after glacial acetic acid treatment were more variable, however. In several instances no change in the enzyme activity was noted with N<sub>2</sub> after this treatment. Similar results on the effect of N<sub>2</sub> were obtained for liver preparations from normal and fasted rats. When smaller amounts of tissue were used (50 mg.) the effect of N<sub>2</sub> was consistent as determined with both sample

<sup>3</sup> We are indebted to Dr. A. L. Lehninger for his suggestions on these experiments.

treatments. Data to illustrate these observations are shown in Table IV. One of the most striking differences observed due to sample treatment was as follows: air ( $\text{H}_2\text{SO}_4$ ) 36.6  $\gamma$ ;  $\text{N}_2$  ( $\text{H}_2\text{SO}_4$ ) 10.0  $\gamma$ ; air ( $\text{CH}_3\text{COOH}$ ) 178  $\gamma$ ; and  $\text{N}_2$  ( $\text{CH}_3\text{COOH}$ ) 190  $\gamma$ . Since quinolinic acid is more highly oxidized than 3-hydroxyanthranilate, traces of oxygen present may have been sufficient for the transformation to occur. No reduction in the con-

TABLE IV

*Effect of  $\text{N}_2$  Atmosphere on Production of Nicotinic Acid Derivatives*

1 mg. of 3-hydroxyanthranilate, 300 mg. of homogenized tissue per flask; 2 hours incubation.

Gas phase	Nicotinic acid produced per flask	
	$\text{N H}_2\text{SO}_4$ treatment	$\text{CH}_3\text{COOH}$ treatment
	$\gamma$	$\gamma$
Air	29	136
	35	202
$\text{N}_2$	14.4	183
	14.4	188
Air*	41.6	226
	38.6	206
$\text{N}_2^*$	11.5	184
	10.0	34
Air	29	125
$\text{N}_2$	6.1	121
	3.5	121
Air†	18.1	112
$\text{N}_2$	4.8	35
Air*†	47.2	238
$\text{N}_2^*\dagger$	11.5	57

\* Tissue from rats fasted for 48 hours.

† 50 mg. of homogenized liver per flask.

version was observed with  $\text{N}_2$  when liver preparations from fasted rats were used (Table IV).

These results, in addition to those observed when the incubation time was varied, emphasize the variable nature of the end-products produced by this system.

It should be emphasized that the conversion has been calculated with nicotinic acid as the end-product, but with the recognition that quinolinic acid is apparently one of the major products and is a precursor of compounds possessing nicotinic acid activity for the test organism. Thus, assuming quinolinic acid as the end-product, the amounts produced are increased by a factor of 1.36. The high activity of the system is readily apparent,

as with the conditions used conversions of 3-hydroxyanthranilate to quinolinic acid up to 50 per cent of theoretical on a molar basis have been observed.

These studies have emphasized the complexity of the enzyme system, and further work with a more purified enzyme system will aid in establishing the cofactor requirements and the nature of the end-products produced from 3-hydroxyanthranilate and other intermediates in the metabolism of tryptophan to nicotinic acid and related compounds.

#### SUMMARY

The conversion by liver slices and homogenates of 3-hydroxyanthranilate to nicotinic acid-active compounds after acid treatment has been studied.

Investigations on the nature of the end-products indicated that considerable quantities of quinolinic acid were produced by this system. A greater amount of compounds possessing nicotinic acid activity for the test organism, *Lactobacillus arabinosus*, was produced by glacial acetic acid treatment than by  $N H_2SO_4$  treatment. Other studies showed that relatively little free or bound nicotinic acid as such was produced.

A reduction in the conversion of 3-hydroxyanthranilate occurred when  $N_2$  was used as the gas phase. This was consistent when  $N H_2SO_4$  treatment was used but was not observed in all cases after glacial acetic acid treatment. None of several inhibitors used decreased the conversion.

The enzymic nature of the system was studied with respect to tissue concentration, time of incubation, etc.

The studies conducted indicate that variable amounts of nicotinic acid-active compounds are produced in certain tests by this system after glacial acetic acid or  $N H_2SO_4$  treatment and emphasize the complex nature of the substances produced.

The reaction, assuming quinolinic acid as the end-product, is very active as measured after glacial acetic acid treatment (up to 50 per cent conversion of added 3-hydroxyanthranilate on a molar basis).

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## BIOCHEMICAL STUDIES OF VIRUS REPRODUCTION

### II. CHEMICAL COMPOSITION OF *ESCHERICHIA COLI* BACTERIOPHAGE T<sub>4</sub> AND ITS HOST\*

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Chemical analysis (1-3) and physicochemical studies (4-6) of closely related *Escherichia coli* bacteriophages have posed several fundamental problems regarding the nature of bacterial viruses: (a) T<sub>2</sub> phage is reported to differ in stability, size, and chemical composition, depending upon whether the source is nutrient broth or synthetic medium lysates (4, 5). The greater size and enhanced lability of phage from the latter medium have been attributed to a coating of desoxyribonucleic acid (DNA) (2, 7): (b) In the stability range T<sub>2</sub> and T<sub>4</sub> coliphages sediment with one or two boundaries according to the pH or Ca<sup>++</sup> content of the solution (4-6). (c) Disagreements exist as to whether the P of T<sub>2</sub> phage is wholly accounted for as desoxyribonucleic acid or whether ribonucleic acid (RNA) and other phosphorylated compounds are also present (1-3, 8, 9).

The first paper in this series (6) described the preparation and properties of *E. coli* bacteriophage T<sub>4</sub>r<sup>+</sup>.<sup>1</sup> Study of this virus in the ultracentrifuge and in the electron microscope has established its similarity in size and morphology to bacteriophage T<sub>2</sub> (6, 9). We have previously reported (6) an electrophoretic analysis of T<sub>4</sub> bacteriophage which revealed that preparations of phage obtained from broth or synthetic medium contained a single major component and that this had the same pH mobility curve. Whereas phage prepared from broth medium usually had a single moving boundary, phage from synthetic medium contained a small amount of rapidly migrating material, which has now been isolated and identified as DNA. This suggests that the apparent differences between phage preparations from the two media are due primarily to adsorbed DNA.

By use of a modified procedure of differential centrifugation described herein, it has now been possible to prepare T<sub>4</sub> bacteriophage which is electrophoretically homogeneous over the whole pH stability range (pH 5.0 to 8.6) and which is apparently free of host antigen. The purified

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<sup>1</sup> The term r<sup>+</sup>, to be understood hereafter for T<sub>4</sub> phage, denotes the wild type strain which forms small plaques on overnight incubation at room temperature (10).



virus sediments with a single sharp boundary at pH 4.9 to about 6.5. Depending on the age of the material and the ionic environment, two ultracentrifugal components are observed at pH 6.5 or higher. This phenomenon will be the subject of a later communication.

This paper presents the results of chemical analysis of bacteriophage T<sub>1</sub> prepared from nutrient broth and from synthetic medium lysates. For purposes of comparison, analyses are given for the bacterial host cultured on these media.

#### EXPERIMENTAL

*Materials and Methods*—The bacteriophage was grown by infection of nutrient broth or synthetic medium cultures of *E. coli* and was purified and assayed for activity by methods previously described (6). The supercentrifuge was used for preliminary concentration of the phage from lysates from both types of media. In the first experiments differential centrifugation at  $2000 \times g$  and  $20,000 \times g$  was employed for purification of the supercentrifuge concentrates of broth phage, while ethanol fractionation was used with the synthetic medium phage (6). In later work the synthetic medium phage was purified by differential centrifugation at  $2000 \times g$  for 15 minutes and  $10,000 \times g$  for 1 hour. Less destruction of the virus occurred at the lower speed, and impurities having sedimentation constants of 300 and 400 Svedberg units were eliminated by this procedure. Synthetic medium phage prepared by this method was essentially homogeneous in the electrophoresis cell and in the ultracentrifuge.

Electrophoresis was carried out as already described (6). Sedimentation rates were measured in the Spinco electrically driven analytical ultracentrifuge,<sup>2</sup> the rotor of which has a diameter of 6.5 cm. as measured to the center of the cell. Photographs were taken at intervals of 2 minutes at a rotor speed of 12,590 R.P.M.<sup>3</sup>

#### Chemical Analysis

*Elementary Analysis*—A purified preparation of phage (infectivity about  $10^{-15.9}$  gm. of N per plaque-forming particle) was dialyzed against distilled water and lyophilized. The poor solubility of the lyophilized product made it suitable only for elementary analysis. The N content of lyophilized phage, dried to constant weight over P<sub>2</sub>O<sub>5</sub>, was 13.2 per cent by the micro-Kjeldahl method and 13.44 per cent by the Dumas method.<sup>4</sup>

<sup>2</sup> Model E, Specialized Instruments Corporation, Belmont, California.

<sup>3</sup> We are indebted to Dr. E. S. G. Barron of the Department of Medicine, University of Chicago, for making available to us the electrophoresis apparatus and the ultracentrifuge used in this investigation, and to Mr. Eugene Goldwasser for performing the electrophoretic analysis.

<sup>4</sup> Dumas N analysis by Dr. J. F. Alicino, Metuchen, New York.

All other analyses reported herein were obtained with saline or buffered solutions of phage, for dialysis against water is reported to disrupt a limiting membrane-like structure with possible loss of normal phage constituents (4, 8).<sup>5</sup>

*Nucleic Acid Analysis of Whole Phage*—The question of the possible presence of RNA in T<sub>6</sub> bacteriophage was examined both by direct colorimetric analysis of whole phage for nucleic acids and by chemical fractionation of virus P. However, for reasons considered in the "Discussion," the majority of analyses was made by the latter procedure with confirmatory nucleic acid color determinations on the separated fractions. DNA was determined by the specific cysteine method of Stumpf (11) by using the Coleman spectrophotometer or Evelyn photoelectric colorimeter at 490 mμ. DNA, prepared from thymus by the method of Hammarsten (12), was employed as a standard, its concentration being determined by P analysis with the conversion factor 10.1. Ribonucleic acid was estimated by the orcinol method of Mejbaum (13) with ribose or a commercial preparation of ribonucleic acid as a standard. The Gomori modification of the Fiske-Subbarow method was used for P analysis (14).

*Phosphorus Partition of Phage*—The P distribution of purified phage in solution was measured by a combination of the partition methods of Schneider (15) and of Schmidt and Thannhauser (16). Both methods give values for acid-soluble P and alcohol-soluble P (hereafter designated fractions I and II). The former procedure separates the phosphoprotein (or residue P) (fraction IV) from a fraction (III) containing RNA and DNA. The latter method precipitates DNA (fraction III') and yields a fraction (IV') containing RNA plus phosphoprotein. By difference two values are obtained for RNA (*i.e.* IV' - IV, and III - III') and, unless otherwise indicated, the average is given in the Tables II to IV. When possible, the RNA content of the separated fractions was also determined by the orcinol method and DNA estimated by the cysteine method. The limitations of partition procedures yielding RNA as a value obtained by difference were recognized, as was the possibility that this fraction might contain metaphosphate or other phosphate. The latter was excluded in several samples by demonstration that the rate of color development in the orcinol reaction of the reputed RNA fraction was similar to that of ribose-3-phosphate (17).<sup>6</sup>

<sup>5</sup> The value for the N content given above thus may not include acid-soluble N, which amounts to about 6 per cent of the total N.

<sup>6</sup> While the procedures employed for separation of the nucleic acids from other P compounds were first developed for mammalian tissues, they have already been applied to bacteria by several authors (1, 2). Their applicability to phage is indicated by the fact that the N:P ratio of the nucleic acid fraction obtained by hot trichloroacetic acid extraction in the Schneider procedure was 1.68, in good agreement with the N:P ratio found for highly purified DNA (18).

### Results

**Identification of Impurity in Synthetic Medium Phage**—The impurity in early preparations of synthetic medium phage  $T_6$ , which migrated with a high mobility nearly independent of pH ( $-16 \times 10^{-5}$  cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup>), was isolated by electrophoretic separation and identified as DNA. Four experiments, all with synthetic medium phage purified by ethanol fractionation, were carried out in the 11 ml. separation cell at pH 5.2, 6.4, 7.6, and 8.6, respectively. For the latter two experiments photographs of the boundaries just before separation are given in Fig. 1. The fast

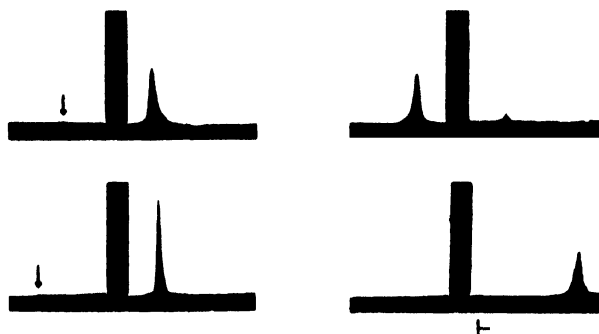


FIG. 1. Electrophoretic purification of *E. coli* bacteriophage  $T_6$  in the 11 ml. separation cell. Upper diagrams, synthetic medium phage purified by ethanol precipitation and electrolyzed at pH 7.60 for 21,000 seconds at 1.22 volts per cm. Lower diagrams, the same at pH 8.58 after 10,000 seconds at 4.11 volts per cm. The horizontal arrows indicate the direction of migration; left ascending (A), right descending (D). The tail of the arrow signifies the position of the starting boundary. Vertical arrows denote a fast moving impurity identified as DNA (see the text). The vertical bar represents the sliding glass surfaces dividing the middle section of the cell into two compartments.

moving component, indicated by the arrows, was isolated in the ascending limb, the slow moving component in the descending limb.

The ultraviolet light absorption spectrum of the isolated fast moving component on the ascending side was characteristic of nucleic acids, with a maximum at 260  $m\mu$  and a minimum at 240  $m\mu$ . In three instances material sufficient for analysis was obtained, giving the following values for DNA by the specific cysteine method: 11, 50, and 51  $\gamma$  per ml. The color in the orcinol reaction could be nearly accounted for by the content of DNA. In the one instance in which sufficient material was obtained for analysis for both types of nucleic acid as well as for direct P analysis, the ratio of DNA P to total P was 0.98. On the basis of these data, as well as from the electrophoretic mobility (6), the fast moving component was identified as DNA. On the basis of infectivity measurements and

P content per virus particle the slow moving component isolated on the descending side was identified as bacteriophage (see Table I).

*Chemical Analysis of T<sub>6</sub> Bacteriophage from Synthetic Medium*—Previous ultracentrifuge studies of bacteriophages (4, 5) have utilized the ultraviolet light absorption method which is less sensitive for the detection of heterogeneity than the schlieren method. An electrically driven analytical ultracentrifuge equipped with a schlieren optical system became available to us only after preparation of broth phage had been discontinued. P partition analyses have, however, been made on three large samples of synthetic medium phage which yielded single sharp sedimenting boundaries in NaCl-acetate buffer at pH 5.0 and 5.8. Sedimentation velocity diagrams of these preparations are shown in Fig. 2. Electrophoretic patterns given in Fig. 3 indicate the absence of impurities

TABLE I

*Properties of Electrophoretically Separated Fast and Slow Fractions of Ethanol Concentrates of E. coli Bacteriophage T<sub>6</sub>*

Material		pH of electrophoretic separation		
		pH 5.18	pH 7.60	pH 8.58
Titer, phage per ml.	Original	$1.6 \times 10^{12}$	$2.1 \times 10^{12}$	$3.0 \times 10^{12}$
	Fast component	$1.9 \times 10^{10}$	$1.1 \times 10^9$	$3.1 \times 10^9$
	Slow "	$2.4 \times 10^{11}$	$3.9 \times 10^{11}$	$5.1 \times 10^{11}$
Infectivity, gm. of N per phage	Original	$10^{-15.92}$	$10^{-15.8}$	$10^{-16.1}$
	Fast component	$10^{-15.35}$		
	Slow "	$10^{-15.84}$		
P per phage, $\gamma \times 10^{11}$	Original	4.5	7.4	4.6
	Fast component	28	100, ca.	187

such as non-constituent DNA. It should be pointed out, however, that these preparations yield two sedimenting boundaries ( $s_{20}$  = about 800 and  $s_{20}$  = about 1050 Svedberg units) on standing in either physiological saline, pH 6.5, or in saline-veronal buffer, pH 7.4 to 8.6. This double boundary phenomenon has previously been observed for T<sub>2</sub> phage (4, 5), and a sedimentation velocity analysis of the phenomenon for T<sub>6</sub> phage will be reported in detail later. In all, some thirty ultracentrifuge analyses have been made of the three preparations in a study of the effects of pH, ionic environment, age, and concentration on the sedimentation behavior of coliphage T<sub>6</sub>. The two preparations also studied in electrophoresis were apparently homogeneous over the pH stability range (about pH 5.0 to 8.6).<sup>7</sup> Moreover, precipitin ring tests with serial 2-fold dilu-

<sup>7</sup> In this paper the term homogeneity is used to indicate the presence of a single boundary in the ultracentrifuge or in the electrophoresis cell. Deviations from more rigid criteria of homogeneity will be considered elsewhere.

tions of the phage against pooled specific rabbit antisera to the host *E. coli*, strain B, indicated the absence of normal bacterial antigenic components.

Prior to phosphate analysis the phage was submitted to prolonged dialysis with stirring in order to reduce any adsorbed inorganic phosphate.

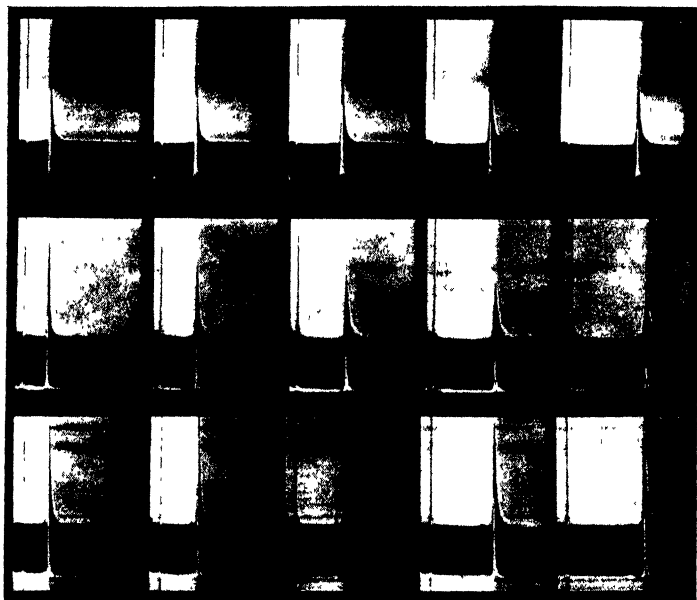


FIG. 2. Ultracentrifuge diagrams of bacteriophage  $T_4$  from synthetic medium. The upper series represent a pooled lot of bacteriophage, 1.85 mg. of phage per ml. in 0.15 ionic strength NaCl-Na acetate buffer, pH 5.0. Middle series, lot XXIII-A, 1.47 mg. of phage per ml. in the same buffer. Lower series,  $N^{15}$ -labeled bacteriophage (lot  $N^{15}$ -I), 2.0 mg. of phage per ml., in unbuffered 0.15 M NaCl, pH about 6.5. All the photographs were taken at intervals of 2 minutes at a mean centrifugal field of 11,400  $g$ . In the diagrams sedimentation proceeds from left to right, and the light-absorbing region due to opalescence of the virus accompanies the sedimenting boundary.

The hot trichloroacetic acid extraction was performed twice. The results of the analyses are given in Table II. Comparison of these figures with earlier results indicated that non-DNA P was diminished by the improved purification procedure and by the intensive dialysis and repeated acid extraction. The percentages of RNA P, residue P, and acid-soluble organic P and ribose were small, and varied from preparation to preparation. However, the ribose and RNA values were determined independently by the orcinol test, and the presence of DNA in the Schmidt and

Thannhauser fraction IV' was excluded by the absence of the specific cysteine reaction for DNA. In these preparations the molar ratio of acid-soluble organic phosphate to ribose was about 1. The rate of hydrolysis of the ribose phosphate in these fractions was similar to that found for ribose-3-phosphate by the method of Albaum and Umbreit (17).

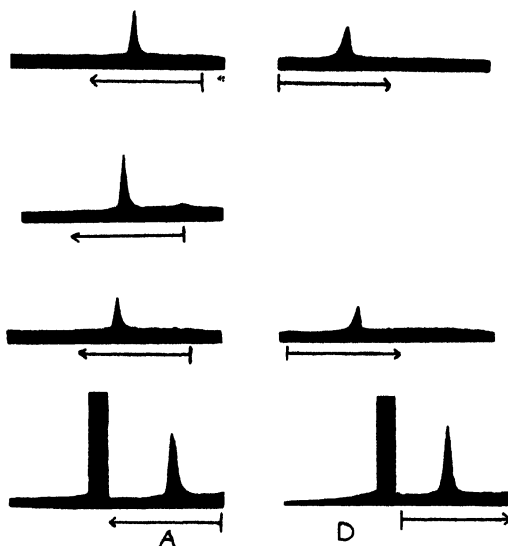


FIG. 3. Electrophoretic diagrams of bacteriophage  $T_4$  from synthetic medium. Upper three photographs,  $N^{15}$ -labeled bacteriophage (lot  $N^{15}$ -I) after electrophoresis in 0.1 ionic strength NaCl-cacodylate or NaCl-veronal buffers of pH 6.58, 8.00, and 8.60, respectively. Lower photograph, bacteriophage lot XXIII-A, after electrophoresis in 0.2 ionic strength Na acetate buffer, pH 5.50. The horizontal arrows indicate the direction of migration; left ascending, right descending. The tail of the arrow signifies the starting boundary. The rapidly migrating component shown in Fig. 1 was absent in these experiments. Although the photographs were taken by infra-red light, a light-absorbing region migrates with the phage in concentrated solutions, resulting in lessened exposure and an elevated and uneven base-line on the solution side of the boundary. The screening effect due to the opalescence has been eliminated in photographic reproduction.

It has not been possible to ascertain whether the inorganic P is adsorbed at the surface of the virus particle or whether it represents an internal constituent. Phage recovered from the electrophoretic separation cell still contains significant amounts of inorganic P.

Two of the preparations of phage for which data are given in Table II ( $N^{15}$ -I and  $N^{15}$ -II) were obtained in the course of experiments on the precursors of virus nucleic acid and protein, with the  $N^{15}$  isotope. The details of the isotope experiments will be presented in a later communica-

tion, but the results of analytical and physicochemical characterization of the purified phage are given below. A yield of 125 ml. of purified phage with a titer of  $2.4 \times 10^{12}$  phage per ml., and an infectivity of  $10^{-15.95}$  gm. of N per phage corresponding to a total of 250 mg. of virus were obtained in the first experiment (N<sup>15</sup>-I). As indicated in Figs. 2 and 3, the purified phage gave a single boundary both in electrophoresis and in the ultracentrifuge. Two separate P partition analyses were performed, one on an aliquot containing 80 mg. of phage. In the latter instance the distribution of N among the several fractions was also deter-

TABLE II  
*Distribution of P and N in E. coli Bacteriophage T<sub>6</sub> from Synthetic (Lactate) Medium\**

Fraction	Pooled phage	Lot XXIII-A	Lot N <sup>15</sup> -I			Lot N <sup>15</sup> -II
	<i>per cent P</i>	<i>per cent P</i>	<i>per cent P</i>	<i>per cent N</i>		<i>per cent N</i>
Acid-soluble (total)	1.6	3.3	0.9	0.7	5.9	5.6
(inorganic)	(0.7)	(1.0)	(0.2)			
Alcohol-soluble	1.2	0.5	0.8	0.5	0.3	
DNA	93.0	91.8†	94.8	97	51.2	51.7
RNA	2.6	3.4‡	2.4			
Residue	1.8	0.7	0.9	1.8	42.7	42.8
Infectivity, gm. N per phage	$10^{-15.89}$	$10^{-15.94}$	$10^{-15.95}$			$10^{-15.92}$
Titer, phage per ml.	$1.9 \times 10^{12}$	$1.7 \times 10^{12}$	$2.4 \times 10^{12}$			$3.6 \times 10^{12}$

\* The column headings refer to preparation numbers. For ultracentrifuge and electrophoresis diagrams, see Figs. 2 and 3. The values for each fraction represent the per cent of total phage P or N. The figures for inorganic P in parentheses are included in total acid-soluble P.

† The ratio of DNA P to total P = 0.93.

‡ Positive orcinol test for RNA; negative cysteine test for DNA.

mined and is given in Table II, 6th column. In this experiment the N:P ratio of the combined nucleic acids (Schneider, fraction III) was 1.74, close to the theoretical value of 1.7 (15, 18). Almost 6 per cent of the N appeared in the acid-soluble fraction.

*Chemical Analysis of Broth Bacteriophage T<sub>6</sub>*—All analyses of concentrates of broth phage were made prior to the acquisition of an ultracentrifuge. Some of the samples were homogeneous in electrophoresis but, because of the absence of ultracentrifugal characterization, the analytical figures for broth phage are considered less reliable than those for phage from synthetic medium.

The DNA content of whole broth phage, as estimated by the cysteine

TABLE III  
Distribution of P in *E. coli* Bacteriophage T<sub>4</sub> from Broth Cultures\*

Type of P	Preparation No.								Average	Broth bacteriophage 1:1	
	XVI†		XVII†	X		III		IX			VI
	per cent	P <sub>n</sub> :P	per cent	per cent	P <sub>n</sub> :P	per cent	P <sub>n</sub> :P	per cent			per cent
Acid soluble-total P	2.65		3.6	7.0		2.0		2.9	1.0	3.2 ± 2.0	
(Inorganic)	(0.6)					(0.6)			(0.2)		
Alcohol-soluble	1.2		1.1	1.0		2.0		2.6	1.2	1.5 ± 0.2	0
DNA	83.8	0.94	84.2	81.5	0.99	82.0	0.92	82.0	88.7	83.5 ± 2.5	82.0
RNA	7.1	0.83	11.0	5.6	0.80	8.9		6.8	5.8	7.0 ± 1.1	12.7
Residue	5.1			5.0		4.9		5.7	3.4	4.8 ± 0.2	5.4
Infectivity, gm. N per phage	10 <sup>-10.0</sup>		10 <sup>-11.8</sup>	10 <sup>-15.87</sup>		10 <sup>-15.7</sup>		10 <sup>-15.8</sup>		10 <sup>-15.3</sup>	10 <sup>-15.9</sup>
P per phage, γ (× 10 <sup>11</sup> )	3.3			4.8				3.4	4.2	3.9	

\* The values for each fraction represent the per cent of total phage P. The figures for inorganic P in parentheses are included in total acid-soluble P. The figures in bold-faced type give the ratio of DNA or RNA P (Pn) found in that fraction by the cysteine or orcinol reactions to P actually found for the fraction.

Homogeneous in electrophoresis (cf. (6) Figs. 3, A and 4).

Homogeneous in eucaryotes (9) (10) (11) (12) (13) (14) (15) (16) (17) (18) (19) (20) (21) (22) (23) (24) (25) (26) (27) (28) (29) (30) (31) (32) (33) (34) (35) (36) (37) (38) (39) (40) (41) (42) (43) (44) (45) (46) (47) (48) (49) (50) (51) (52) (53) (54) (55) (56) (57) (58) (59) (60) (61) (62) (63) (64) (65) (66) (67) (68) (69) (70) (71) (72) (73) (74) (75) (76) (77) (78) (79) (80) (81) (82) (83) (84) (85) (86) (87) (88) (89) (90) (91) (92) (93) (94) (95) (96) (97) (98) (99) (100) (101) (102) (103) (104) (105) (106) (107) (108) (109) (110) (111) (112) (113) (114) (115) (116) (117) (118) (119) (120) (121) (122) (123) (124) (125) (126) (127) (128) (129) (130) (131) (132) (133) (134) (135) (136) (137) (138) (139) (140) (141) (142) (143) (144) (145) (146) (147) (148) (149) (150) (151) (152) (153) (154) (155) (156) (157) (158) (159) (160) (161) (162) (163) (164) (165) (166) (167) (168) (169) (170) (171) (172) (173) (174) (175) (176) (177) (178) (179) (180) (181) (182) (183) (184) (185) (186) (187) (188) (189) (190) (191) (192) (193) (194) (195) (196) (197) (198) (199) (200) (201) (202) (203) (204) (205) (206) (207) (208) (209) (210) (211) (212) (213) (214) (215) (216) (217) (218) (219) (220) (221) (222) (223) (224) (225) (226) (227) (228) (229) (230) (231) (232) (233) (234) (235) (236) (237) (238) (239) (240) (241) (242) (243) (244) (245) (246) (247) (248) (249) (250) (251) (252) (253) (254) (255) (256) (257) (258) (259) (260) (261) (262) (263) (264) (265) (266) (267) (268) (269) (270) (271) (272) (273) (274) (275) (276) (277) (278) (279) (280) (281) (282) (283) (284) (285) (286) (287) (288) (289) (290) (291) (292) (293) (294) (295) (296) (297) (298) (299) (300) (301) (302) (303) (304) (305) (306) (307) (308) (309) (310) (311) (312) (313) (314) (315) (316) (317) (318) (319) (320) (321) (322) (323) (324) (325) (326) (327) (328) (329) (330) (331) (332) (333) (334) (335) (336) (337) (338) (339) (340) (341) (342) (343) (344) (345) (346) (347) (348) (349) (350) (351) (352) (353) (354) (355) (356) (357) (358) (359) (360) (361) (362) (363) (364) (365) (366) (367) (368) (369) (370) (371) (372) (373) (374) (375) (376) (377) (378) (379) (380) (381) (382) (383) (384) (385) (386) (387) (388) (389) (390) (391) (392) (393) (394) (395) (396) (397) (398) (399) (400) (401) (402) (403) (404) (405) (406) (407) (408) (409) (410) (411) (412) (413) (414) (415) (416) (417) (418) (419) (420) (421) (422) (423) (424) (425) (426) (427) (428) (429) (430) (431) (432) (433) (434) (435) (436) (437) (438) (439) (440) (441) (442) (443) (444) (445) (446) (447) (448) (449) (450) (451) (452) (453) (454) (455) (456) (457) (458) (459) (460) (461) (462) (463) (464) (465) (466) (467) (468) (469) (470) (471) (472) (473) (474) (475) (476) (477) (478) (479) (480) (481) (482) (483) (484) (485) (486) (487) (488) (489) (490) (491) (492) (493) (494) (495) (496) (497) (498) (499) (500) (501) (502) (503) (504) (505) (506) (507) (508) (509) (510) (511) (512) (513) (514) (515) (516) (517) (518) (519) (520) (521) (522) (523) (524) (525) (526) (527) (528) (529) (530) (531) (532) (533) (534) (535) (536) (537) (538) (539) (540) (541) (542) (543) (544) (545) (546) (547) (548) (549) (550) (551) (552) (553) (554) (555) (556) (557) (558) (559) (560) (561) (562) (563) (564) (565) (566) (567) (568) (569) (570) (571) (572) (573) (574) (575) (576) (577) (578) (579) (580) (581) (582) (583) (584) (585) (586) (587) (588) (589) (590) (591) (592) (593) (594) (595) (596) (597) (598) (599) (600) (601) (602) (603) (604) (605) (606) (607) (608) (609) (610) (611) (612) (613) (614) (615) (616) (617) (618) (619) (620) (621) (622) (623) (624) (625) (626) (627) (628) (629) (630) (631) (632) (633) (634) (635) (636) (637) (638) (639) (640) (641) (642) (643) (644) (645) (646) (647) (648) (649) (650) (651) (652) (653) (654) (655) (656) (657) (658) (659) (660) (661) (662) (663) (664) (665) (666) (667) (668) (669) (670) (671) (672) (673) (674) (675) (676) (677) (678) (679) (680) (681) (682) (683) (684) (685) (686) (687) (688) (689) (690) (691) (692) (693) (694) (695) (696) (697) (698) (699) (700) (701) (702) (703) (704) (705) (706) (707) (708) (709) (710) (711) (712) (713) (714) (715) (716) (717) (718) (719) (720) (721) (722) (723) (724) (725) (726) (727) (728) (729) (730) (731) (732) (733) (734) (735) (736) (737) (738) (739) (740) (741) (742) (743) (744) (745) (746) (747) (748) (749) (750) (751) (752) (753) (754) (755) (756) (757) (758) (759) (760) (761) (762) (763) (764) (765) (766) (767) (768) (769) (770) (771) (772) (773) (774) (775) (776) (777) (778) (779) (780) (781) (782) (783) (784) (785) (786) (787) (788) (789) (790) (791) (792) (793) (794) (795) (796) (797) (798) (799) (800) (801) (802) (803) (804) (805) (806) (807) (808) (809) (810) (811) (812) (813) (814) (815) (816) (817) (818) (819) (820) (821) (822) (823) (824) (825) (826) (827) (828) (829) (830) (831) (832) (833) (834) (835) (836) (837) (838) (839) (840) (841) (842) (843) (844

§ Data calculated from (1).

**Includes inorganic P.**



method, averaged 42 per cent. The ratio of DNA P to total P approached unity. However, chemical fractionation of the phage revealed the presence of acid-soluble P and ribose, as well as of RNA. In the results given in Table III the per cent of DNA was relatively constant, but markedly lower than that found for phage from synthetic medium (Table II). For comparative purposes the results of Taylor (1) for T<sub>2</sub> broth phage are included in Table III.

Electrophoretic analysis indicated that free desoxyribonucleic acid is not present as an impurity in these preparations. The marked variation in acid-soluble organic P and the apparently higher content of RNA in broth phage are to be noted. Incubation of a sample of phage (lot XVI) with trypsin, followed by repurification and chemical fractionation, had little effect on the phosphate distribution of broth phage (see Table

TABLE IV  
*Distribution of P in E. coli from Broth and Synthetic Media*

Type of P	Broth bacteria*	Synthetic medium bacteria†
	per cent	per cent
Acid-soluble	20.7 ± 2.4	24.6 ± 1.4
Alcohol-soluble	11.1 ± 0.9	15.7 ± 5.0
RNA	48.5 ± 1.9	38.4 ± 4.8
DNA	13.1 ± 0.1	22.6 ± 3.5
Phosphoprotein	5.2 ± 3.0	4.4 ± 0.6
RNA P:DNA P	3.5 ± 0.1	1.8 ± 0.4

\* Average of five analyses.

† Average of four analyses

III, 3rd column). The P of the precipitated DNA fraction is nearly accounted for as desoxyribonucleic acid by the cysteine reaction. Although the P content of the separated RNA fraction was obtained by difference, and only 80 per cent of the P appeared to be combined as ribonucleic acid, orcinol color tests were sufficient to exclude DNA as the chief P component of this fraction.

*Phosphorus Partition of Bacterial Host*—The results of P partition of *E. coli*, strain B, grown in nutrient broth or synthetic medium, are presented in Table IV. The bacteria were obtained from aerated actively growing cultures. For analytical purposes 100 ml. of culture containing about  $2 \times 10^8$  cells per ml. were employed. The bacterial suspensions were chilled, centrifuged in the cold, and washed three times with saline before P partition. Comparison of the ratio of RNA P:DNA P reveals a striking difference between bacteria from the two media. The ratio of

RNA P:DNA P for bacteria grown in broth (3, 5) is similar to the value of 3.68 reported by Taylor (1). The ratio for bacteria cultured in synthetic medium (1.8) differs markedly from the figure of 8.35 calculated from Taylor's data. However, the latter author used glucose rather than lactate as a source of carbon for the synthetic medium, and it is well known that the chemical composition of *E. coli* depends on the age of the bacteria and the conditions of growth.

#### DISCUSSION

In several recent reviews on the nature of animal and bacterial viruses, considerable significance has been attached to the results of chemical analysis of the purified agents (2, 8, 19). Some viruses have been found to consist only of nucleoproteins, while other larger viruses have appeared to contain several additional constituents. Question has arisen, especially with regard to the possible presence of both RNA and DNA in bacteriophage  $T_2$  (1-3) and in some animal viruses (8, 19, 20).<sup>3</sup> In a few instances no criteria of virus purity have been presented; in others, the marked limitations of the colorimetric methods of nucleic acid analysis have been overlooked.

In this investigation attention was directed first towards the isolation and identification of a rapidly migrating impurity originally discovered by electrophoretic analysis (6). Previous study of the action of desoxyribonuclease in diminishing the viscosity of concentrates of bacteriophages  $T_2$  and  $T_4$  from synthetic medium had suggested that up to 30 per cent of the total DNA was present at the surface of the virus particle (7). In agreement, it has been found by planimetric measurement of the areas in electrophoretic diagrams that freely migrating DNA comprised up to 25 per cent of the total nucleic acid of some preparations of synthetic medium phage. In considering the nature and origin of the external DNA, Cohen (7) has first reported that "the stability of  $T_2$  as a function of DNA . . . and the different sizes of  $T_2$  in broth and simple medium according to Hook *et al.* strongly suggest a structural rôle of this material." However, he later stated (2) that "it is difficult to say to what extent these effects are due to decomposition products of  $T_2r^+$ ." In view of the fact that the DNA is electrophoretically separable, the first hypothesis now

<sup>3</sup> The several authors also disagree in the elementary analytical figures. For example, for synthetic medium  $T_2$  bacteriophage Cohen and Anderson (21) report an N content of 11.84 per cent and a P content of 3.66 per cent (the latter corresponding to 36.9 per cent DNA). However, Taylor (1) found 13.3 per cent N and 5.22 per cent P, and a DNA content of 44.6 per cent. Such discrepancies may be attributed to the lack of demonstration of homogeneity of the phage on the part of some authors and to the presence of non-constituent DNA or variable amounts of inorganic phosphate.

seems untenable. Further, since the impurity is best identified by electrophoretic rather than by ultracentrifugal study, chemical analysis of bacteriophage concentrates not submitted to both types of characterization may be subject to a large correction for non-constituent DNA.

The second possible impurity and a potential source of RNA in bacteriophage concentrates are bacterial debris liberated during lysis. With impure preparations of  $T_6$  phage from synthetic medium, up to four ultracentrifugal components have been observed, with sedimentation constants of about 300, 400, 800, and 1000 Svedberg units. The latter two components predominate and represent the phage; the former are removed by the modified procedure of differential centrifugation which yields virus that is ultracentrifugally monodisperse at pH 5 to 6.5. Although impure preparations of  $T_6$  bacteriophage gave positive precipitin ring tests with anti-*E. coli* rabbit sera, no evidence of bacterial antigen was found for several of the ultracentrifugally homogeneous virus preparations submitted to chemical analysis. Such studies are unavailable for  $T_2$  bacteriophage.

One object of this investigation was to determine whether *E. coli* bacteriophages contain both RNA and DNA, as reported by Taylor (1) for  $T_2$  phage, or exclusively DNA, as claimed by Cohen (2, 3) for the same phage. Chemical fractionation of the virus phosphorus indicated that RNA P accounted for only 2.4 to 3.4 per cent of the total P of the most highly purified preparations of synthetic medium  $T_6$  bacteriophage obtained in this study and for 2.3 per cent of the total P of synthetic medium  $T_2$  bacteriophage analyzed by Taylor.<sup>9</sup> Such low values obtained by difference of the P content of other fractions cannot be considered sufficiently reliable to establish the presence of small amounts of RNA in a preparation rich in DNA, even when partially substantiated by the orcinol color test on the separated RNA-containing fraction. On the other hand, the single criterion used by Cohen for the exclusion of RNA as a constituent of bacteriophage is likewise inadequate, for the observation that the ratio of DNA P determined by the diphenylamine reaction to total P

<sup>9</sup> Higher values for RNA P were found by chemical fractionation of  $T_6$  phage from broth lysates, and, it is to be noted that, while these were obtained by difference, they were substantially confirmed by the orcinol reaction (Table III). Similarly, Taylor (1) reported a higher proportion of RNA P in  $T_2$  phage from broth lysates and concluded that the chemical composition of  $T_2$  phage depends on the culture medium. In this investigation, because of the lack of ultracentrifuge characterization of phage from broth medium, no conclusions have been drawn as to the significance of the differences in chemical composition found for  $T_6$  phage from broth and synthetic media.

is 0.99 rests on the doubtful assumption that the proportion of purines in the phage and in the standard thymus nucleic acid is the same.<sup>10</sup>

The data on the chemical composition of *E. coli* and T<sub>2</sub> bacteriophage presented herein affirm the striking difference in the distribution of the P constituents of the parasite and its host (1). This is in contrast to the apparent similarity in amino acid composition of T<sub>4</sub> bacteriophage and *E. coli* suggested by a preliminary report (23). As Beard has pointed out (8), infection of a unicellular host by the bacteriophages affords a singular opportunity for study of the relation of virus constitution to that of its host. The data from chemical analysis are in accord with isotope experiments which indicate that virus reproduction involves an accelerated synthesis of desoxyribonucleic acid; for the DNA content of the bacterial cell is insufficient to account for the DNA found in the phage liberated by lysis (3, 9). A study of the origin of virus P and of the fate of the infecting virus particle will be presented elsewhere. Experiments with the N<sup>15</sup> isotope for further investigation of the mode of virus reproduction are now in progress.

#### SUMMARY

A rapidly migrating impurity found in some preparations of *Escherichia coli* bacteriophage T<sub>2</sub> has been isolated by electrophoretic separation and identified as desoxyribonucleic acid (DNA). The differences in phage obtained from broth and synthetic medium are attributed to this non-constituent DNA, which is probably adsorbed at the surface of the virus but is without structural significance. Electrophoretically and ultracentrifugally homogeneous bacteriophage T<sub>2</sub> has been prepared from synthetic medium lysates by a modified procedure of differential centrifugation. Chemical analysis of the purified phage indicates that the virus consists chiefly of desoxyribonucleoprotein. Although the chemical composition of the bacterial host depends upon the medium of culture, only small differences exist in phage prepared from broth or synthetic medium lysates.

The authors are grateful to Dr. E. A. Evans, Jr., for help and encouragement in this work.

<sup>10</sup> Since diphenylamine reacts only with purine nucleotides, use of this method for the determination of DNA requires either that the proportion of purines in the phage and the standard nucleic acid be the same or that the proportions in both be known and a correction applied (22). Cohen and Anderson (21) report that the ratio of purine desoxyribose to total desoxyribose in T<sub>2</sub> phage is 0.48, while that of standard thymus nucleic acid is 0.50. On the other hand, the most recent analytical data indicate that the molar proportion of total purines to total pyrimidines in thymus DNA is 1.2 (18).

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# FATTY ACID SYNTHESIS BY ENZYME PREPARATIONS OF CLOSTRIDIUM KLUYVERI\*

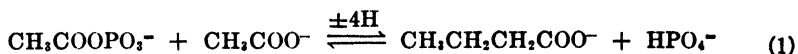
## V. A CONSIDERATION OF POSTULATED 4-CARBON INTER- MEDIATES IN BUTYRATE SYNTHESIS

BY E. R. STADTMAN AND H. A. BARKER

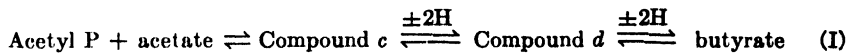
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It has been shown (11, 12) that enzyme preparations of the bacterium *Clostridium kluveri* catalyze the reversible conversion of acetyl phosphate and acetate to butyrate (equation (1)).



This conversion has been postulated to involve a preliminary condensation of acetyl phosphate and acetate to a 4-carbon compound (Compound c, Scheme I) which is the primary oxidant in the reaction, and which is reduced in two steps to butyrate.



Compound c should be in a state of oxidation corresponding to acetoacetate, and Compound d, the second postulated intermediate, should be in the same state of oxidation as crotonate,  $\beta$ -hydroxybutyrate, or vinyl acetate.

It has been shown that enzyme preparations of *C. kluveri* can utilize molecular hydrogen as the reductant in the conversion of acetyl phosphate and acetate to butyrate (12), and that they can utilize molecular oxygen as the oxidant for the reverse process, the oxidation of butyrate to acetyl phosphate and acetate (11). These enzyme preparations therefore provide an excellent test system to determine whether postulated intermediates are involved in butyrate synthesis. Thus, any substance that is a normal intermediate should be oxidized, in the presence of oxygen, to acetyl phosphate and acetate and the rate of oxidation should be as great as or greater than the corresponding oxidation of butyrate. In a hydrogen atmosphere an intermediate should be reduced to butyrate at a rate comparable to the reduction of acetyl phosphate and acetate to butyrate. Finally, the rate of oxygen or hydrogen uptake with the intermediates alone should be the

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same as with a mixture of the intermediate and butyrate or acetyl phosphate and acetate, respectively.

With these considerations in mind, studies were made to determine whether several substances that have been postulated as intermediates are actually involved in butyrate synthesis.

Acetoacetate, postulated as the 4-carbon Compound *c* in Scheme I, has already been studied extensively and shown not to be a normal intermediate in the synthesis or oxidation of butyrate (13). Other 4-carbon compounds in a state of oxidation corresponding to acetoacetate have not been investigated.

However, substances corresponding to Compound *d* in Scheme I have been investigated and the results will be reported here. Of the 4-carbon compounds tested, vinyl acetate is the only substance readily attacked by the enzyme preparations. Vinyl acetate is decomposed in a manner consistent with the view that it is a normal intermediate in the synthesis and oxidation of butyrate. Tracer studies, however, have shown definitely that vinyl acetate is not an intermediate.

#### *Materials and Methods*

Vinylacetic acid was prepared by the procedure described by Blatt (1). Duclaux distillation values of this acid were found to be only slightly higher than those obtained for acetic acid; therefore, it is difficult to differentiate between these two acids by the rate of distillation from aqueous solution. A quantitative separation of these acids was easily obtained, however, by partition chromatographic analysis, by the procedure of Elsdén (4). Vinylacetic acid is eluted from the hydrated silica gel column at a rate intermediate between the rates of butyric and acetic acids.

Isocrotonate was obtained through the courtesy of D. E. Green. Crotonate was obtained from the Eastman Kodak Company.

All other methods and the enzyme preparations used in this investigation have been described previously (10–12).

#### *Results*

*Crotonate and Isocrotonate*—In sharp contrast to the behavior of animal preparations (5–7, 9), the enzyme preparations of *C. kluyveri* were found to be completely ineffective in either oxidizing or reducing crotonate or isocrotonate. Attempts to stimulate oxidation or reduction of these compounds by additions of acetyl phosphate, adenylic acid, adenosine triphosphate, inorganic phosphate, or yeast extract gave entirely negative results. These compounds appear, therefore, to be completely inert so far as the enzyme preparations are concerned and may be excluded as intermediates in butyrate synthesis.

*$\beta$ -Hydroxybutyrate*—It has already been shown that acetoacetate is reduced quantitatively to  $\beta$ -hydroxybutyrate and that a further reduction of  $\beta$ -hydroxybutyrate to butyrate does not occur even when a source of high energy phosphate (acetyl phosphate, adenosine triphosphate) is available. Therefore,  $\beta$ -hydroxybutyrate cannot be a normal intermediate in butyrate synthesis.

### *Studies with Vinyl Acetate*

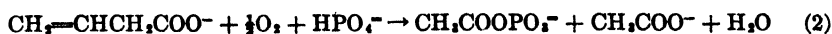
*Oxidation of Vinyl Acetate to Acetyl Phosphate and Acetate*—When vinyl acetate was shaken with the enzyme preparation in air, oxygen was consumed rapidly. Data of a typical experiment are presented in Table I.

TABLE I  
*Aerobic Oxidation of Vinyl Acetate*

Each Warburg vessel contained 2.0 ml. of 0.1 M inorganic phosphate buffer (pH 8.1) and the indicated amounts of enzyme preparation and vinyl acetate. The samples were incubated in air for 95 minutes at 26°, at which time the O<sub>2</sub> uptake had ceased in the sample to which 17.6  $\mu$ M of vinyl acetate had been added. Oxygen uptake had not stopped in the other vessel, although the rate had declined to one-half the initial rate. The data have been corrected for oxygen uptake (1.2  $\mu$ M, Lot G; 2.6  $\mu$ M, Lot H) and for acetyl phosphate (1.2  $\mu$ M, Lot G; 2.3  $\mu$ M, Lot H) in control samples to which no substrate was added.

Enzyme preparation	Vinyl acetate added	-O <sub>2</sub>	$\Delta$ acetyl P	$\frac{\Delta \text{ acetyl P}}{-\text{O}_2}$
	$\mu$ M	$\mu$ M	$\mu$ M	$\mu$ M
20 mg. cell-free extract (Lot G) . . . . .	17.6	7.8	15.2	1.95
50 " dry cells (Lot H) . . . . .	88	28	54	1.93

For each mole of oxygen consumed, 2 moles of acetyl phosphate were formed. The data are therefore consistent with the view that vinyl acetate was oxidized quantitatively to acetyl phosphate and acetate (equation (2)).



*Reduction of Vinyl Acetate to Butyrate*—In a similar experiment (Table II), vinyl acetate was incubated with the enzyme preparation in a hydrogen atmosphere. Under these conditions, hydrogen was consumed and an almost equivalent molar quantity of butyrate was formed. The data may be represented by equation (3).



*Rate of Oxidation and Reduction of Vinyl Acetate*—The above experiments show qualitatively that vinyl acetate satisfies some of the requirements of



an intermediate in butyrate synthesis; *i.e.*, it is oxidized to acetyl phosphate and reduced to butyrate. Further studies were made to determine whether the rates of oxidation and reduction were sufficiently high to account for butyrate oxidation and synthesis.

It was found that vinyl acetate was oxidized 15 to 20 per cent more rapidly than butyrate. A mixture of vinyl acetate and butyrate was oxidized at the same rate as vinyl acetate alone. The failure to observe a greater rate of oxidation with the mixture cannot be regarded as proof that the same enzyme is responsible for the oxidation of both vinyl acetate and butyrate, since some factor other than dehydrogenase activity may have limited the rate of oxygen uptake. It was not possible to increase the rate of oxygen uptake by the addition of other oxidizable substrates such as ethanol. Nevertheless, the fact that vinyl acetate is oxidized more rapidly

TABLE II  
*Reduction of Vinylacetic Acid with H<sub>2</sub>*

The Warburg vessel contained 100  $\mu\text{M}$  of inorganic phosphate (pH 8.1), 20 mg. of cell-free extract (Lot G), and 17.6  $\mu\text{M}$  of vinyl acetate. The total volume was 2.0 ml. The gas phase was H<sub>2</sub>. The substrate was tipped into the enzyme solution after the enzyme was saturated with H<sub>2</sub> (100 minutes). The sample was then incubated 3 hours at 26°. All data have been corrected for small blank values obtained in a control sample to which no substrate was added. The butyrate content was computed from Duclaux distillation data.

Initial vinyl acetate	-H <sub>2</sub>	$\Delta$ butyrate	$\Delta$ volatile acids	$\Delta$ butyrate -H <sub>2</sub>
$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$	
17.6	10.1	12.3	+1.6	1.2

than butyrate is consistent with the view that vinyl acetate is an intermediate in the oxidation of butyrate.

In another experiment (Table III) the reduction of vinyl acetate was compared with the reduction of a mixture of acetyl phosphate and acetate. The rates of hydrogen uptake were the same for both samples. However, the rate of hydrogen uptake for a mixture of acetyl phosphate, acetate, and vinyl acetate was about 25 per cent lower than for vinyl acetate alone. This inhibitory effect, though small, is readily reproducible and is certainly significant. It is not due to a higher absolute concentration of reducible substrates in the mixture, since similar inhibition is observed at lower concentrations also. The fact that vinyl acetate is reduced to butyrate as rapidly as a mixture of acetyl phosphate and acetate may be purely fortuitous. This result also may be due to a rate-limiting hydrogen-activating system. All attempts to increase the rate of hydrogen uptake by the

addition of other reducible substrates such as butyryl phosphate<sup>1</sup> were unsuccessful. The lower rate of hydrogen uptake for a mixture of vinyl acetate, acetyl phosphate, and acetate is not readily explained on the basis of the idea that vinyl acetate is a normal intermediate in butyrate reduction. A further discussion of the effect will be deferred, since it is more easily explained in the light of other experimental results to be described.

*Anaerobic Oxidation and Dismutation of Vinyl Acetate*—According to equation (2), 1 mole of oxygen oxidizes 2 moles of vinyl acetate. Results at variance with this were obtained from isotope experiments to be described. For example, it was found in one experiment that all of the vinyl acetate disappeared before an equivalent amount of oxygen was consumed.

TABLE III

*Reduction of Vinyl Acetate and Acetyl Phosphate Plus Acetate with Molecular Hydrogen*

Each Warburg vessel contained 0.05 M inorganic phosphate (pH 8.0), 50 mg. of cell-free extract (Lot G), and, where indicated, 40  $\mu$ M of acetyl phosphate, 175  $\mu$ M of acetate, and 44  $\mu$ M of vinyl acetate. The total liquid volume was 2.0 ml. The gas phase was hydrogen. Temperature, 26°. The substrates were dumped from the side arms of the vessels after the enzyme was saturated with hydrogen (60 minutes).

Incubation time	Hydrogen consumed		
	Acetyl P + acetate	Vinyl acetate	Vinyl acetate + acetyl P + acetyl
	$\mu$ .	$\mu$ .	$\mu$ .
5	35	36	26
10	66	66	48
20	127	127	92
30	192	190	136
40	248	243	176

This observation suggested that vinyl acetate might have undergone a dismutation, 1 molecule being oxidized to acetyl phosphate and acetate, while another was reduced to butyrate (equation (4)).

To test this possibility, experiments were made in which vinyl acetate was incubated with the enzyme preparation in a nitrogen atmosphere. The results presented in Table IV show that, during the 2 hour incubation period, 23  $\mu$ M of hydrogen and 57  $\mu$ M of acetyl phosphate were formed and there was a net increase in volatile acids of 62  $\mu$ M. A Duclaux distillation of the volatile acid fraction indicated the presence of considerable amounts of other more steam-volatile fatty acids. Chromatographic analysis of the

<sup>1</sup> It has been shown that enzyme preparations of *C. kluyveri* catalyze the reduction of butyryl phosphate to butanol in a hydrogen atmosphere (unpublished data).

fatty acids obtained from another sample incubated under the above conditions showed that appreciable amounts of butyrate and some caproate were formed. Thus, as was anticipated, the vinyl acetate was decomposed anaerobically and acetyl phosphate and butyrate were formed. Very unexpected, however, was the additional observation that hydrogen was produced.

These results indicate that under anaerobic conditions two types of reactions occur. There is a dismutation of vinyl acetate in which acetyl phosphate, acetate, and butyrate are formed (equation (4)), and also an

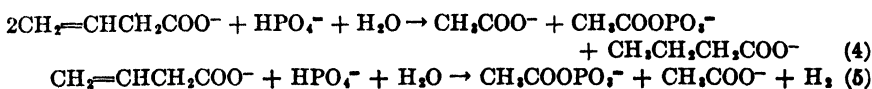
TABLE IV

*Anaerobic Oxidation and Dismutation of Vinyl Acetate*

The Warburg vessel contained 50 mg. of dried cells (Lot G) and 200  $\mu\text{M}$  of inorganic phosphate (pH 8.1). The gas phase was  $\text{N}_2$  (99.5 per cent). The center well contained 0.2 ml. of 10 per cent KOH. The substrate was mixed with the enzyme after equilibration at 26°. The mixture was incubated at this temperature until no further  $\text{H}_2$  production was observed (2 hours).

Initial vinyl acetate	$\text{H}_2$ formed	$\Delta$ acetyl P	Final volatile acid	$\Delta$ volatile acid
$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$
176	23	57	238	62

anaerobic oxidation of vinyl acetate occurs to form acetyl phosphate, acetate, and hydrogen (equation (5)).



The data of Table IV show a close agreement between the amount of acetyl phosphate formed and the net increase in volatile acid, as is to be expected from equations (4) and (5). This indicates that 62  $\mu\text{M}$  of vinyl acetate were decomposed to acetyl phosphate and acetate. Since 23  $\mu\text{M}$  of hydrogen were formed, at least 23  $\mu\text{M}$  of vinyl acetate were oxidized by way of reaction (5). This means that 39  $\mu\text{M}$  (62 - 23 = 39) of acetyl phosphate, acetate, and butyrate were formed by dismutation. In other words, 78  $\mu\text{M}$  of the vinyl acetate were decomposed by dismutation. The dismutation of vinyl acetate was therefore approximately 3 times as rapid as the anaerobic oxidation giving hydrogen.

*Influence of Arsenate and Inorganic Phosphate on Oxidation and Reduction of Vinyl Acetate*—It has been shown that no oxidation of butyrate occurs in the absence of inorganic phosphate or in the presence of inorganic arsenate. Preliminary studies with vinyl acetate have shown that this substance also is not oxidized aerobically in the absence of inorganic phos-

phate or in the presence of 0.025 M arsenate. However, a small but significant reduction of vinyl acetate by hydrogen does occur in the presence of arsenate.

*Vinyl Acetate As Intermediate in Synthesis or Oxidation of Butyrate*—The experiments described thus far are consistent with the view that vinyl acetate is an intermediate in the synthesis of butyrate. The evidence in favor of this conclusion may be summarized as follows: (1) Vinyl acetate is rapidly oxidized aerobically to acetyl phosphate and acetate. The rate of this oxidation is slightly greater than the corresponding oxidation of butyrate. (2) Vinyl acetate is reduced to butyrate by molecular hydrogen. The rate of hydrogen uptake is as rapid as in the reduction of acetyl phosphate plus acetate; therefore the rate of butyrate formation is twice as great. (3) Vinyl acetate undergoes a rapid dismutation in which 1 molecule is reduced to butyrate and 1 molecule is oxidized to acetyl phosphate and acetate. (4) Vinyl acetate undergoes an anaerobic oxidation in which hydrogen, acetyl phosphate, and acetate are formed. This may be a reversal of the first reduction step in the conversion of acetyl phosphate and acetate to butyrate. (5) Finally, vinyl acetate, like butyrate, is not oxidized in the presence of arsenate or in the absence of inorganic phosphate.

On the other hand, at variance with these lines of evidence is the observation that a mixture of vinyl acetate, acetyl phosphate, and acetate is reduced to butyrate more slowly than is vinyl acetate alone (Table III).

It was desirable, therefore, to obtain more definite information regarding the actual intermediary formation of vinyl acetate in the conversion of acetyl phosphate and acetate to butyrate and in the oxidation of butyrate. Since vinyl acetate is so readily converted to acetyl phosphate and acetate and butyrate, it seemed unlikely that sufficient quantities would accumulate to enable one to isolate and identify it. However, a definitive test of vinyl acetate formation could be made by appropriate use of the isotope tracer technique. For example, if carboxyl-labeled acetate and acetyl phosphate are reduced to butyrate in the presence of an excess of unlabeled vinyl acetate, the vinyl acetate should become labeled if it is an obligatory intermediate in the oxidation of butyrate; moreover, the final activity of the vinyl acetate should be as great as or greater than the final activity of the butyrate. Likewise, if vinyl acetate is an obligatory intermediate in the oxidation of butyrate, the oxidation of labeled butyrate to acetate in the presence of unlabeled vinyl acetate should cause the vinyl acetate to become labeled, and its specific activity should be equal to or greater than that of the acetate.

In view of these considerations, the following isotope experiments were performed.

*Aerobic Oxidation of Carboxyl-Labeled Butyrate and Unlabeled Vinyl*

*Acetate*—Butyrate (42  $\mu\text{M}$ ) that was labeled in the carboxyl group with  $\text{C}^{14}$  and unlabeled vinyl acetate (88  $\mu\text{M}$ ) were shaken with the enzyme preparation in air. After 33  $\mu\text{M}$  of oxygen were consumed, the experiment was discontinued. The volatile acids were fractionated by partition chromatography, and radioactivity measurements were made on the individual acids. Butyric and acetic acids, identified by Duclaux distillation, were the only acids present.

No vinyl acetate could be found after incubation, even though the total amount of oxygen consumed (33  $\mu\text{M}$ ) was sufficient to have oxidized only 75 per cent of the vinyl acetate added (equation (2)). Moreover, part of the oxygen consumed was probably used to oxidize butyrate. Thus the complete disappearance of vinyl acetate indicated that it could be decomposed by an anaerobic reaction. This was later confirmed by experiments already described. Radioactivity measurements on the residual

TABLE V

*Aerobic Oxidation of Carboxyl-Labeled Butyrate in Presence of Ordinary Vinyl Acetate*

50 mg. of dry cells (Lot G) in 2.0 ml. of 0.1 M inorganic phosphate buffer (pH 8.1) were incubated with the substrates indicated, in air, at 26°.

Compound		Total	Total counts per min.	Counts per min. per micromole
		$\mu\text{M}$		
Before incubation	Butyrate	42	117,000	2800
	Vinyl acetate	88	0	0
After incubation (95 min.)	Butyrate	31	33,700	1080
	Vinyl acetate	0		
	Acetate	165	72,500	440

butyrate and on the acetate that had been produced are presented in Table V. The data show that the specific activity of the residual butyrate was only about 40 per cent as great as that of the added butyrate. This proves that an appreciable amount of butyrate was formed from vinyl acetate. It does not prove, however, that there was a net production of butyrate from vinyl acetate; a similar decrease of the specific activity would be obtained if the vinyl acetate and butyrate were interconvertible.

*Anaerobic Oxidation and Dismutation of Vinyl Acetate in Presence of Carboxyl-Labeled Butyrate and Unlabeled Acetate*—To determine whether an equilibrium was established between butyrate and vinyl acetate, a mixture of carboxyl-labeled butyrate, unlabeled acetate, and vinyl acetate was incubated in a Warburg vessel in an atmosphere of nitrogen. Under these conditions hydrogen production could be measured and would give a direct measure of the amount of vinyl acetate that was converted directly to acetate and acetyl phosphate by reaction (5). After incubation the fatty

acids were separated chromatographically. The results of  $C^{14}$  measurements on the various fatty acids are presented in Table VI.

The specific activity of the butyrate decreased about 20 per cent during incubation, showing that about 20  $\mu M$  of butyrate had been formed. However, the specific activity of the residual vinyl acetate was only 4.4 counts per minute per micromole, or about 1 per cent as great as that of the residual butyrate. It is obvious, therefore, that very little equilibration had taken place between butyrate and vinyl acetate in spite of the fact that an appreciable amount of butyrate had been produced from vinyl acetate or acetate.

TABLE VI

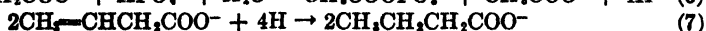
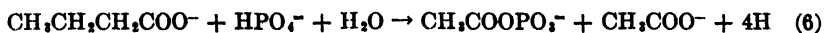
*Anaerobic Oxidation and Dismutation of Vinyl Acetate in Presence of Carboxyl-Labeled Butyrate and Unlabeled Acetate*

50 mg. of dry cells (Lot G) in 2.0 ml. of 0.1 M inorganic phosphate buffer were incubated with the substrate in an  $N_2$  atmosphere at 28°.

Compound		Total	Total counts per min.	Counts per min. per micromole
		$\mu M$		
Before incubation	Butyrate	97	54,300	560
	Vinyl acetate	176	0	0
	Acetate	75	0	0
After incubation (45 min.)	Butyrate	93	40,700	438
	Vinyl acetate	133	600	4.4
	Acetate + acetyl P	116	3,200	28
	Caproate	8	(3,500)*	(438)*
	$H_2$	7.3		

\* Radioactivity measurements on the caproate were not made. In other almost identical experiments, it was found that the specific activity of the caproate was slightly higher than that of the butyrate.

The specific activity of the residual acetate was 28 counts per minute per micromole, which was several times greater than the specific activity of the vinyl acetate. This shows quite conclusively that butyrate was oxidized to acetate without going through vinyl acetate. Since no oxygen was present, the only added oxidant was vinyl acetate. It appears, therefore, that butyrate is oxidized to acetyl phosphate and acetate by a path that does not pass through vinyl acetate, and this oxidation is coupled with a direct reduction of vinyl acetate to butyrate (equations (6) and (7)).



The net result is the formation of 1 mole of inactive butyrate, 1 mole of acetyl phosphate, and 1 mole of radioactive acetate.

The total activity in the acetate fraction (free acetate plus acetyl phosphate) was 3200 counts per minute, from which it can be estimated that about 7  $\mu\text{M}$  of butyrate were oxidized by reactions (6) and (7). However, the total increase in the acetate fraction was 40 to 50  $\mu\text{M}$ , indicating that about 30 to 40  $\mu\text{M}$  were formed directly from the vinyl acetate. Since 7.3  $\mu\text{M}$  of hydrogen were formed, part of the acetate probably came directly from the anaerobic oxidation of vinyl acetate by reaction (5), and the rest must have been formed by a dismutation of the vinyl acetate (reaction (4)).

Actually the system is too complicated to analyze completely on the basis of the available experimental data. Explanations other than those presented are possible. A further investigation of the reactions of vinyl acetate is desirable. Nevertheless, two important conclusions are possible from the available data. (a) A rapid equilibration between vinyl acetate and butyrate does not occur; the reduction of vinyl acetate to butyrate must therefore be practically irreversible under the experimental conditions we have used. (b) Vinyl acetate is not an obligatory intermediate in the oxidation of butyrate to acetyl phosphate and acetate.

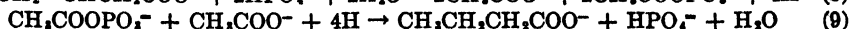
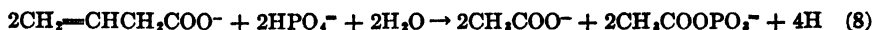
*Reduction of Carboxyl-Labeled Acetate and Acetyl Phosphate to Butyrate in Presence of Unlabeled Vinyl Acetate*—In view of the above conclusions, it seemed very unlikely that vinyl acetate could be an intermediate in the synthesis of butyrate from acetyl phosphate and acetate.

To exclude this possibility completely, an experiment was designed in which acetate labeled in the carboxyl group with  $\text{C}^{14}$  would be reduced to butyrate in the presence of unlabeled vinyl acetate. The most obvious way to do such an experiment would be to add acetyl phosphate and use hydrogen as a reductant. However, the use of hydrogen appeared to be objectionable because of the possibility that vinyl acetate would be rapidly and preferentially reduced to butyrate. It was decided, therefore, to use vinyl acetate as the only reductant. No acetyl phosphate was added, because this compound was known to be formed rapidly from vinyl acetate (equation (2)). At the end of the incubation period 46  $\mu\text{M}$  of acetyl phosphate were found in the reaction mixture. The experiment was carried out in a nitrogen atmosphere and the course of the reaction was followed manometrically by observing hydrogen evolution (equation (5)).

The results of radioactivity measurements are given in Table VII. The final specific activity of the acetate was only 40 per cent as great as the initial activity. This was to be expected, since there was more than a 2-fold increase in the total acetate concentration. The butyrate formed (28  $\mu\text{M}$ ) had a specific activity of 47 counts per minute per micromole, but no radioactivity could be detected in the residual vinyl acetate. This proves conclusively that vinyl acetate is not an obligatory intermediate in the synthesis of butyrate from acetate.

An interesting parallelism exists between the anaerobic oxidation of

butyrate to acetyl phosphate in the presence of vinyl acetate and the anaerobic oxidation of vinyl acetate in the presence of acetate and acetyl phosphate. In the former instance vinyl acetate acts as the oxidant and is presumably reduced to butyrate (equations (6) and (7)), while in the latter instance vinyl acetate is the reductant and is oxidized to acetyl phosphate and acetate (equations (8) and (9)).



The discovery that acetate and acetyl phosphate were reduced to butyrate by reactions (8) and (9) suggested the possibility that all of the butyrate produced from vinyl acetate was formed in this manner. In this event, it would be unnecessary to postulate that a direct reduction of vinyl acetate

TABLE VII

*Anaerobic Oxidation of Vinyl Acetate in Presence of Carboxyl-Labeled Acetate*

50 mg. of dry cells (Lot G) in 2.0 ml. of 0.1 M inorganic phosphate buffer (pH 8.1) were incubated in an  $\text{N}_2$  atmosphere at 26°.

Compound		Total	Total counts per min.	Counts per min. per micromole
		$\mu\text{M}$		
Before incubation	Acetate	80	22,200	277
	Vinyl acetate	176	0	0
After incubation (45 min.)	Acetate + acetyl P	170	18,500	109
	Acetyl P	46		
	Vinyl acetate	85	0	0
	Butyrate	28	1,300	47
	Caproate	5	287	58
	$\text{H}_2$	9		

to butyrate occurs. Examination of the data in Table VII shows, however, that reactions (8) and (9) can account for a relatively small amount of the butyrate formed. For example, the total activity of the butyrate was 1300 counts per minute. The average specific activity of the acetate during the experiment was 193 counts per minute per micromole, which means that the butyrate formed from acetate would have an average specific activity of  $2 \times 193 = 386$  counts. Thus the total amount of butyrate derived from acetate would be  $1300/386 = 3.4 \mu\text{M}$ . Actually 28  $\mu\text{M}$  of butyrate were produced, indicating that over 20  $\mu\text{M}$  were formed by direct reduction of vinyl acetate.

#### DISCUSSION

Systematic investigations of the rôle of various 4-carbon compounds in the synthesis or oxidation of butyrate have been made before. Thus



Davies (3) found that cell suspensions and growing cultures of *Clostridium acetobutylicum* were unable to convert numerous 4-carbon compounds (including the ones tested above) to butyrate. His experiments could not, however, be considered conclusive, since the possibility remained that the negative results were due to the inability of the substances to penetrate the cell walls.

The inability to achieve a synthesis of butyrate *in vitro* with animal preparations has made it impossible to obtain clear cut results regarding the rôle of postulated intermediates in butyrate synthesis in animals. However, tissue slices and particulate enzyme preparations of animals have been obtained that catalyze the oxidation of butyrate to acetoacetate. These preparations in general have been very non-specific in their ability to catalyze the oxidation of 4-carbon compounds to acetoacetate (5-7, 9), and a clear cut result regarding the rôle of these compounds as intermediates in the normal process of butyrate oxidation has not always been obtained.

In contrast to the animal studies, the complete lack of enzymatic activity on crotonate, isocrotonate, and  $\beta$ -hydroxybutyrate of enzyme preparations of *C. kluyveri* definitely excludes these substances as normal intermediates in the synthesis or oxidation of butyrate.

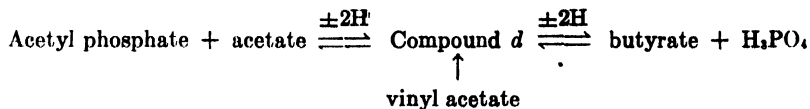
Kleinzeller (7), using rat liver and kidney slices, made a systematic study of the utilization of several 4-carbon fatty acids in an effort to determine the identity of intermediates in butyrate oxidation. Of the substances tested, vinyl acetic acid was the only one oxidized more rapidly than butyrate, and it was concluded that vinyl acetate could be an intermediate. Grafflin and Green (5) have obtained similar results with particulate suspensions prepared from rabbit liver and kidney tissues. Lehninger (8), working with similar particulate suspensions prepared from rat liver, concluded that vinyl acetate was not an intermediate in the formation of acetoacetate from octanoate. The failure of vinyl acetate to be attacked by *C. acetobutylicum* (3) could also be taken as evidence that vinyl acetate is not a normal intermediate in the synthesis of butyrate.

Studies on the decomposition of vinyl acetate by the enzyme preparations of *C. kluyveri* are therefore of particular interest, since they tend to reconcile the diverse opinions regarding the possible rôle of this substance in butyrate synthesis. Vinyl acetate is readily oxidized to acetyl phosphate and acetate and it is readily reduced to butyrate, but isotope experiments have offered convincing evidence that it is not a normal intermediate in the oxidation or synthesis of butyrate.

Evidence is available from the tracer experiments to show that the oxidation of butyrate to acetyl phosphate and acetate can occur under anaerobic conditions if the oxidation is coupled with the reduction of vinyl acetate to butyrate (equations (6) and (7)). Similarly, the reduction of acetyl

phosphate and acetate to butyrate may be coupled with the oxidation of vinyl acetate to acetyl phosphate and acetate (equations (8) and (9)). Yet, in neither the oxidation of butyrate nor the reduction of acetyl phosphate and acetate to butyrate is vinyl acetate formed as an intermediate.

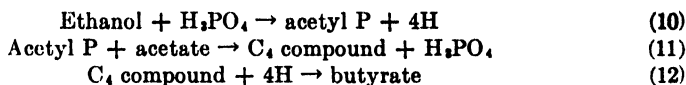
The most reasonable explanation for this rather anomalous situation appears to be that vinyl acetate is converted by means of a relatively irreversible reaction into a substance (Compound *d*) that is on the main pathway in the synthesis and oxidation of butyrate.



Other possibilities are not excluded. For example, vinyl acetate could be reduced directly to butyrate and oxidized to acetyl phosphate and acetate without going through a common intermediate. In this event, both the oxidation and reduction reactions would have to be relatively irreversible.

The discovery that vinyl acetate can serve as the reductant in the conversion of acetyl phosphate and acetate to butyrate permits a reasonable explanation for the observation that the hydrogen uptake by a mixture containing vinyl acetate, acetate, and acetyl phosphate is less rapid than with a mixture of acetyl phosphate and acetate only (Table III). In the presence of an excess of acetyl phosphate and acetate, the vinyl acetate may compete with molecular hydrogen as a reductant, and hence the lower hydrogen uptake observed in the mixture.

The data available from the enzyme studies on *C. kluyveri* (11, 12) show that the synthesis of butyrate from ethanol and acetate is accomplished by the following generalized mechanism (equations (10), (11), (12)).



Although the over-all conversion of ethanol and acetate to butyrate is an exergonic process involving the liberation of 11.7 kilocalories (2), the data obtained thus far indicate that this conversion involves the formation of but one energy-rich phosphate bond (reaction (10)) and that this phosphate bond energy is utilized in effecting the condensation of acetyl phosphate and acetate to form a 4-carbon compound (equation (11)) that is the oxidant for ethanol oxidation and is reduced to butyrate (equation (12)). Such a scheme presents the curious situation of the phosphate bond energy generated in the oxidation of ethanol being entirely consumed in forming the oxidant for the reaction. It would therefore appear that the bacterium would be unable to obtain any useful energy in the form of phos-

phate compounds from the process. In other words, the above mechanism provides no way in which there can be a net production of energy-rich phosphate.

The discovery that hydrogen is evolved in the anaerobic oxidation of vinyl acetate is therefore of considerable interest. Such a reaction does lead to a net production of phosphate bond energy in the form of acetyl phosphate (equation (5)). In this reaction the oxidation of vinyl acetate to acetyl phosphate is coupled with the formation of molecular hydrogen that can escape from the system. It may well be that the most of the energy available to *C. kluyveri* for synthesis of cell materials is derived from this or an analogous mechanism. Such a theory gains support from the fact that the growth of *C. kluyveri* is always associated with the production of considerable quantities of molecular hydrogen. Normally about 10 per cent of the ethanol is converted to molecular hydrogen and acetate (or acetyl phosphate) (2).

Bornstein and Barker (2) have pointed out that thermodynamic considerations exclude the possibility that the hydrogen is liberated in the oxidation of ethanol to acetaldehyde and they postulated that the hydrogen was evolved in the oxidation of acetaldehyde to acetate. While the latter reaction is theoretically possible, it would not result in the formation of an energy-rich phosphate. The corresponding oxidation of acetaldehyde to acetyl phosphate with the liberation of hydrogen appears unlikely from thermodynamic considerations.

With enzyme preparations of *C. kluyveri* we have been unable to demonstrate the evolution of hydrogen from either ethanol or acetaldehyde. To date, the only well defined hydrogen-producing reaction shown to be catalyzed by the enzyme preparations is the anaerobic oxidation of vinyl acetate (equation (5)). Although the data obtained from the tracer experiments exclude vinyl acetate as an obligatory intermediate in butyrate synthesis, small but significant amounts of vinyl acetate were formed from butyrate (Table VI). It is therefore tempting to conclude that the hydrogen produced by growing cultures is derived from vinyl acetate or from a related 4-carbon compound, and that this type of reaction is important in supplying energy for growth.

#### SUMMARY

The ability of enzyme preparations of *Clostridium kluyveri* to oxidize and reduce several 4-carbon compounds that have been postulated as intermediates in the synthesis and oxidation of butyrate was studied.

$\beta$ -Hydroxybutyrate, crotonate, and isocrotonate are not oxidized or reduced by the enzyme preparations and may therefore be excluded as intermediates in butyrate synthesis or oxidation.

Vinyl acetate is oxidized to acetyl phosphate and acetate, and is reduced to butyrate. Although the rates of these reactions are consistent with the view that vinyl acetate is an intermediate in the synthesis and oxidation of butyrate, it was shown by tracer studies that vinyl acetate is not an obligatory intermediate.

Under anaerobic conditions (in a nitrogen atmosphere) tracer experiments have shown that vinyl acetate undergoes the following reactions: (a) It undergoes dismutation, 1 molecule being oxidized to acetyl phosphate plus acetate, while another molecule is reduced to butyrate. (b) It is oxidized to acetyl phosphate and acetate with the evolution of hydrogen gas. (c) In the presence of butyrate, the reduction of vinyl acetate is coupled with the oxidation of butyrate to acetyl phosphate and acetate. (d) In the presence of acetyl phosphate and acetate, the oxidation of vinyl acetate is coupled with the reduction of acetyl phosphate and acetate to butyrate.

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# THE PHYSICAL PROPERTIES OF A CRYOGLOBULIN OBTAINED FROM LYMPH NODES AND SERUM OF A CASE OF LYMPHOSARCOMA\*

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During the course of an investigation of the electrophoretic properties of extracts of human neoplastic lymphoid tissues, a distinctly abnormal component was observed in a case of lymphosarcoma. On examining the serum of this patient, it was found that lowering the temperature to 4° caused spontaneous precipitation of a large amount of protein, which redissolved completely when the serum was warmed to room temperature. Further investigation revealed that the abnormal proteins from tissue and serum were identical with respect to electrophoretic mobility and sedimentation constant.

The phenomenon of spontaneous reversible precipitation of blood proteins in the cold has been reviewed by Lerner *et al.* (1) who suggested that these proteins be termed cryoglobulins. Lerner reported that of 121 pathological sera studied by him thirty-one contained a cryoglobulin, while forty normal sera contained none. The diseases associated with the presence of a cryoglobulin both in Lerner's studies and in those previously reported were so varied that the phenomenon could not be considered to be characteristic of any one disease. However, cryoglobulins appear to be somewhat commonly found in cases of myeloma.

Morrison (2) isolated a cryoglobulin from human *plasma* by salting out with  $(\text{NH}_4)_2\text{SO}_4$  at 16.6 per cent saturation. This cryoglobulin, described as a part of the fibrinogen complex, had the property of clotting in the presence of anticoagulants and was thought to be responsible for increased erythrocyte sedimentation rates. No physical constants were determined.

Morrison, Edsall, and Miller (3) in a study of the large scale separation of purified fibrinogen from pooled "normal" human plasma by alcohol fractionation, described a fraction (Fraction I-1) which contained fibrinogen and a non-clottable component having the property of spontaneously precipitating in the cold. This latter component was termed "cold insoluble globulin." Since the amount recovered varied from one run to the next and constituted only 0.3 to 0.7 per cent of the total plasma protein, it was

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felt that the cold insoluble globulin was not a normal blood protein, but was contributed to the pooled plasma by a small percentage of donors.

A detailed investigation of the properties of these apparently pathological cryoglobulins would seem desirable, since such information may provide some insight as to the underlying metabolic defects which give rise to their formation. The detailed characterization of such proteins may further permit studies in the human of some of the factors which are concerned

TABLE I  
*Physicochemical Properties of Cryoglobulins*

Bibliographic reference No.	Source	Electrophoretic mobility, $10^5$ cm. per volt per sec.	Sedimentation constant, $S_{20, w}$	Diffusion constant, $D_{20, w}$ , $10^7$ cm. <sup>2</sup> per sec.	Molecular weight
(4)	Myeloma serum		7.1 (80%)	3.4	200,000
(5, 6)	" " ?		19.1	1.6	1,080,000
(7)	" " ?*	3.4, pH 4.7	6.8		
(8)	Acro purpura serum	3.38, pH 4.7, 0.1 $\mu$ acetate			190,000†
(3)	Plasma Fraction I	" $\beta_1$ "-globulin	13		
This paper	Lymphosarcoma	-2.2, pH 8.6, 0.1 $\mu$ veronal	16 (85%)		
" "	Tissue and serum	+5.38, pH 4.7, 0.02 $\mu$ acetate	23 (15%)	2.6	600,000

\* Precipitated spontaneously at room temperature and had crystalline structure.

† Determined by osmotic pressure.

with the synthesis of a specific protein. Finally, it is important to know whether these proteins, which have in common the property of insolubility at low temperature, have other properties in common. Whether cryoglobulins from different sources prove to be identical or to be a closely related group of proteins, or whether they prove to be a group of widely different proteins with a single property in common, their appearance in diseased states presents a challenge for correlation of the disease process with the appearance of these entities.

We have undertaken the characterization of a cryoglobulin isolated from both the lymph nodes and serum of a case of lymphosarcoma<sup>1</sup> and have compared the findings with those of other investigators who have examined cryoglobulins by similar techniques. Measurements of electrophoretic mobility, sedimentation and diffusion constants, and ultraviolet absorption have been made. Any differences between various cryoglobulins revealed by these procedures would provide evidence of non-identity. A summary

<sup>1</sup> Kuzma, J. F., Laskowski, M., and Hirschboeck, J. S., to be published.

of the physical constants reported for various cryoglobulins is given in Table I. These data indicate that the cryoglobulins studied to date differ widely in properties referable to molecular size and shape. The mobility of cryoglobulin reported in this paper, in veronal buffer at pH 8.6 and 0.1 ionic strength, was approximately the same as the " $\beta_1$ "-globulin reported by Morrison, Edsall, and Miller (3). The mobility measured at pH 4.7 in acetate buffer cannot be compared to those reported by the two other investigators, since in one case (8) the ionic strength was not the same, while in the other (7) the ionic strength was not stated.

#### EXPERIMENTAL

*Isolation of Cryoglobulin from Lymphoid Tissue Extract*—A tissue extract suitable for electrophoretic study was prepared from lymph nodes by the method described previously by the authors (9). Two separate masses of lymph nodes obtained post mortem from different regions were treated separately. They were cut into thin slices and washed by repeated centrifugation and resuspension in 0.07 M  $\text{KHCO}_3$ -0.07 M KCl at approximately pH 8.0. The washed tissue was then homogenized with an equal volume of the same salt solution, and centrifuged at  $15,000 \times g$  for 2 hours. All operations were carried out in a cold room at  $4^\circ$ . The clear supernatant extract was dialyzed to equilibrium against veronal buffer at 0.1 ionic strength, pH 8.6, and electrophoresis was carried out in the same buffer at  $1^\circ$ , with use of the Longworth modification of the Tiselius apparatus. The patterns of the extracts from the two lymphoid masses were almost identical (Fig. 1, *a* and *b*). The large sharply defined peak with a mobility of  $-2.2 \times 10^{-5}$  cm.<sup>2</sup> per volt per second did not correspond to any of the components previously noted in either human normal lymphoid tissue (tonsil) (9) or human pathological lymphoid tissue studied to date.<sup>2</sup>

In order to define more exactly the nature of this unusual protein component, the two extracts were pooled and brought to equilibrium with 0.3 saturated  $(\text{NH}_4)_2\text{SO}_4$  by dialysis. The precipitate which formed was washed with the outside dialysis fluid and taken up in a small volume of potassium bicarbonate-potassium chloride mixture. *Only a part of the precipitate redissolved.* The remainder did not redissolve at  $4^\circ$  until a larger amount of salt solution was added. This more sparingly soluble fraction was examined electrophoretically and found to contain the whole of the pathological protein of the tissue together with some other tissue components. The pattern is shown in Fig. 1, *c*. The sedimentation diagram<sup>3</sup> of this fraction (Fig. 3, *a*) showed one sharp main component ( $s_{20, w} = 16$ ) together with small amounts of poorly resolved heavier and lighter com-

<sup>2</sup> Unpublished results.

<sup>3</sup> Determined in the Spinco ultracentrifuge.



ponents (approximately 29 and 8 Svedberg units respectively). The electrophoretic mobility and sedimentation constants of the principal component were identical with those of a cryoglobulin, described in the next section, isolated from the serum of the same patient.

*Cryoglobulin from Serum*—The serum from the same patient was obtained 20 months before death and preserved in the frozen state. It was clear at room temperature but yielded a heavy precipitate at 4°. This precipitate redissolved completely at room temperature. In order to carry out an electrophoresis experiment at 1°, the serum was diluted 1:4 with veronal

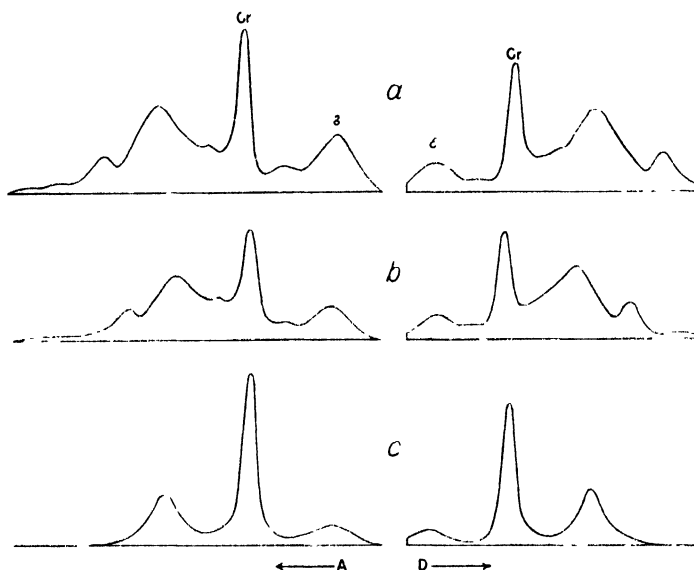


FIG. 1. Electrophoretic patterns of tissue fractions. (a) and (b) whole extract from two separate lymphoid masses. (c)  $(\text{NH}_4)_2\text{SO}_4$  fraction prepared as described in the text. The abnormal component is marked Cr. Electrophoresis for about 140 minutes at 6 volts per cm., in veronal buffer, pH 8.6, 0.1 ionic strength.

buffer; at this dilution, no precipitation at 1° took place. The electrophoretic pattern of the whole serum is shown in Fig. 2, a. The sharp abnormal component had a mobility of  $-2.2 \times 10^{-5}$  cm.<sup>2</sup> per volt per second and constituted 36 per cent of the total area.

The cryoglobulin was separated by cooling the undiluted serum to 4°. The precipitate was separated and washed four times with small amounts of saline at 4°. The electrophoretic pattern (Fig. 2, b) showed that the cryoglobulin is the abnormal component of the whole serum. A small amount of contaminating serum protein was still evident. Further purification was carried out by  $(\text{NH}_4)_2\text{SO}_4$  precipitation at 0.3 saturation and

further washing with small amounts of saline at 4°. The electrophoretic patterns (concentration 0.5 per cent) with veronal buffer at 0.1 ionic

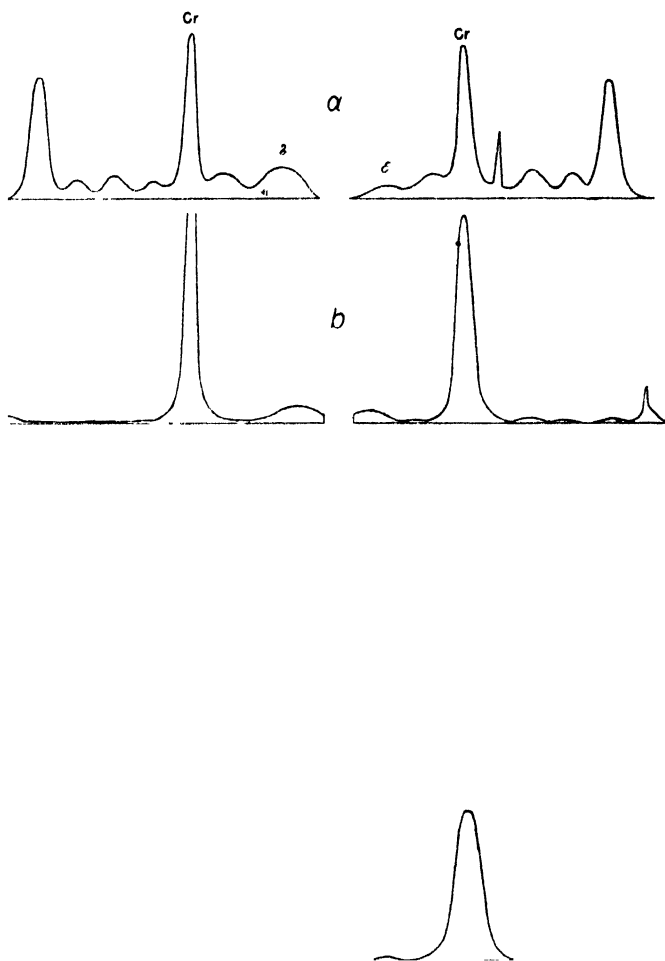


FIG. 2. Electrophoretic patterns of whole serum and purified cryoglobulin. (a) whole serum (1:4). (b) cryoglobulin purified by cold precipitation and washing. (c) cryoglobulin purified further by  $(\text{NH}_4)_2\text{SO}_4$  precipitation. (a), (b), and (c) in veronal buffer, 0.1 ionic strength, pH 8.6, for approximately 150 minutes at 60 volts per cm. (d) same preparation as (c), in acetate buffer, 0.02 ionic strength, pH 4.7, for 85 minutes at 6.4 volts per cm. The cryoglobulin component is marked Cr.

strength, pH 8.6, and acetate buffer at 0.02 ionic strength, pH 4.7, are shown in Fig. 2, c and d. At pH 8.6 the cryoglobulin has a negative net

charge, while at pH 4.7 it has a positive net charge. In each case, the protein migrated as a single symmetrical boundary with no evidence of other components. The mobilities are given in Table I; acetate at 0.02 ionic strength was the salt of the highest concentration in which the protein

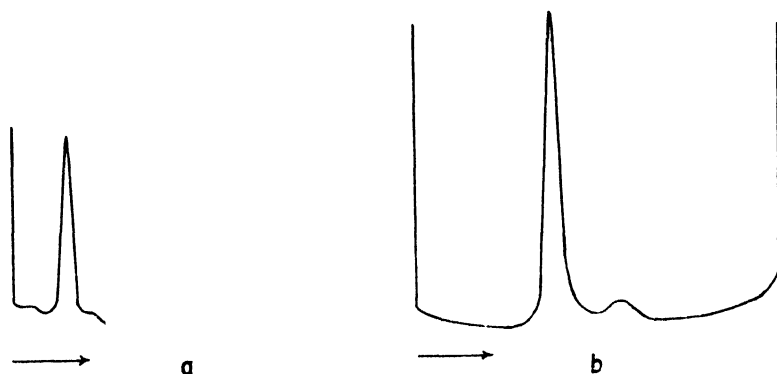


FIG. 3. Sedimentation diagrams of cryoglobulin. (a)  $(\text{NH}_4)_2\text{SO}_4$  fraction from lymph node extract in veronal buffer, 0.1 ionic strength, pH 8.6, after sedimentation for about 10 minutes at  $254,900 \times g$ . (b) purified serum cryoglobulin in veronal buffer, 0.1 ionic strength, pH 8.6, after sedimentation for about 25 minutes at  $125,000 \times g$ . The vertical line at the left indicates the position of the meniscus. The vertical line at the right indicates the bottom of the cell.

TABLE II  
*Sedimentation Constants of Tissue and Serum Cryoglobulin\**

Solvent	Concentration of protein	Source	Sedimentation constant, $s_{20, w}$	
			Major component	Minor component
0.1 $\mu$ NaCl	0.5	Serum	14.9	21.6
0.02 $\mu$ acetate, pH 4.7	0.5	"	15.1	22.0
0.10 $\mu$ veronal, pH 8.6	0.5	"	15.5	20.0
Same	0.25	"	16.0	23.0
"	0.25	Tissue	16.0	8; 29

\* See Fig. 3 for sedimentation diagrams.

was soluble at pH 4.7. In contrast to the electrophoretic analysis, the sedimentation diagram revealed two components (Fig. 3, b), with the main component accounting for 85 per cent and a heavier component accounting for 15 per cent of the total area. Neither the number of components nor the relative areas were significantly affected by change in solvent, pH, or

concentration of protein. The sedimentation constants obtained in different solvents at about 24° and corrected to water at 20° are given in Table II.

A determination of the sedimentation constant at two concentration levels,<sup>4</sup> 0.5 and 0.25 per cent, suggests a small concentration dependence, but not enough determinations at widely differing concentrations were carried out to justify an extrapolation to infinite dilution.

The diffusion constant of the same protein sample in veronal buffer, pH 8.6, 0.10 ionic strength, was determined by the refractometric measurement of free diffusion in the Tiselius cell with the scanning method of Longsworth (10). Analysis of the refraction index gradient curves by means of the maximum height and area method gave a value of  $2.80 \pm 0.06 \times 10^{-7}$  cm.<sup>2</sup> per second corrected to water at 20°. Analysis by means of the half width at the point of inflection gave  $2.44 \pm 0.02 \times 10^{-7}$  cm.<sup>2</sup> per second. The average of the two values is  $2.6 \times 10^{-7}$  cm.<sup>2</sup> per second.

The ultraviolet absorption spectrum was typical for proteins and agreed closely with that determined for the cryoglobulin described by Lerner and Greenberg (8).

#### DISCUSSION

From a consideration of the physical constants and solubility of this pathological protein, it is obvious that it is different from any blood protein, either normal or pathological, which has heretofore been studied (Table I). Furthermore, it would seem from the information at hand that this particular blood cryoglobulin is derived from the cells of lymphosarcoma tissue, since both the serum and tissue extract contain large amounts. Unequivocal proof of the latter point requires the demonstration that the components of the tissue extract were not in part derived from the tissue lymph fluid or contaminating blood. The following considerations make the possibility of contamination with extracellular fluid unlikely: (1) The tissue extract was nearly colorless and thus was not grossly contaminated with whole blood. (2) Thin slices of the tissue were washed with a large volume of bicarbonate-KCl buffer before breaking the cells. (3) Extracts which contained components of nearly identical mobility (Fig. 1, *a* and *b*) were obtained from two separate masses of lymph nodes. (4) The ratio of the concentration of the cryoglobulin component to the "albumin" in the tissue extract was different from that in the serum (3.4 and 1.0 respectively).

Little is known as to the origin of the cryoglobulins. The liver has been reported to be a probable site of formation (1). Although, as pointed out above, the presence of a protein in a tissue extract does not establish that tissue as the site of formation, it seems likely, from the considerations out-

<sup>4</sup> Protein concentration determined by nitrogen analysis and factor of 6.25.

lined, that in this condition the pathological cells are directly involved in the process.

The molecular weight of 600,000 for the main component of the cryoglobulin was calculated from the sedimentation and diffusion constant by means of the Svedberg equation, assuming a partial specific volume of 0.75. The value 600,000 should be considered only approximate, since the diffusion constant was determined for a system containing two components in the ultracentrifuge although homogeneous in electrophoresis. The calculated frictional ratio,  $f/f_0$ , is 1.45, which corresponds to a ratio of major to minor axis of 9 for an ellipsoid of revolution, assuming zero hydration.

The significance, in terms of metabolic abnormality, of the presence of large amounts of a high molecular weight protein in the lymphoid tissue and serum, can at present be only a matter of conjecture. Waldenström (5) advanced the hypothesis that these pathological proteins represent protein newly formed in response to the presence of a virus which acts as a template. Although speculation on this problem is tempting, our present state of knowledge does not permit an adequate explanation of the factors controlling the elaboration of normal plasma proteins and thus precludes a reasonable basis for an explanation for the appearance of abnormal proteins in plasma. It is hoped that the continued study of the intracellular proteins of normal and diseased tissues will provide the information necessary for establishing the relationships of these to the plasma proteins.

#### SUMMARY

The sedimentation constant, diffusion constant, ultraviolet absorption spectrum, and electrophoretic mobility of a cryoglobulin isolated from the lymph nodes and serum of a case of lymphosarcoma have been determined. The protein, electrophoretically homogeneous at pH 4.7 and 8.6, but containing two components of different sedimentation constant, did not completely correspond in its properties to any cryoglobulin previously described.

The significance of the finding of similar abnormal proteins in both serum and lymphatic tissue of the same patient is discussed.

We wish to express our thanks to Dr. J. S. Hirschboeck, Department of Medicine, Marquette University, for providing us with serum from this patient and to Dr. J. F. Kuzma, pathologist, Columbia Hospital, Milwaukee, for providing lymph nodes obtained at autopsy.

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# EFFECT OF PITUITARY EXTRACTS UPON GLUCOSE UPTAKE BY DIAPHRAGMS FROM NORMAL, HYPOPHYSECTOMIZED, AND HYPOPHYSECTOMIZED-ADRENALECTOMIZED RATS\*

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The measurement of the glucose uptake of rat diaphragm muscle *in vitro* is of interest because striated muscle is quantitatively the most important tissue for glucose disposal in the animal body and because glucose uptake, for which the hexokinase reaction is presumably the rate-determining enzymatic step, has been shown to be under hormonal control.

The evidence for hormonal control obtained so far consisted in the demonstration that the glucose uptake by diaphragm from diabetic animals was depressed below normal (1, 2), that it was slightly above normal in adrenalectomized and diabetic-adrenalectomized rats, and that it was markedly increased in diaphragms from hypophysectomized animals (1, 3). The addition of insulin to the diaphragm *in vitro* caused a marked acceleration of uptake under all conditions so far tested.

It has now been found that injection of certain pituitary fractions can depress the glucose uptake by diaphragm from hypophysectomized animals to or below the normal level and the uptake by normal diaphragm to the diabetic level. The participation of adrenal cortical secretions in this effect is indicated by the fact that injections of pituitary extracts do not cause inhibition in hypophysectomized-adrenalectomized animals unless adrenal cortical extract is injected at the same time.

## Methods and Materials

**Animals**—Young male rats of the Sprague-Dawley and Anheuser-Busch strains were used. They were maintained under the conditions previously described (3).

Hypophysectomized animals, weighing about 125 gm. at the time of operation,<sup>1</sup> were males of the Sprague-Dawley strain or, in a few cases,

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<sup>1</sup> The hypophysectomies were performed by the Hormone Assay Laboratories, Chicago.



females of the Anheuser-Busch strain. The rats were used between 15 and 30 days after operation.

Hypophysectomized-adrenalectomized animals were prepared by single stage adrenalectomy of Sprague-Dawley males under sodium hexobarbital anesthesia 15 to 30 days after hypophysectomy. These animals were given 0.5 per cent sodium chloride to drink and remained in good condition until used 48 to 72 hours after operation.

*Test Procedure*—One-half of the excised diaphragm was incubated with shaking for 1 hour at 37° in Krebs-Henseleit salt solution containing 140 mg. per cent of glucose. The other half was incubated under the same conditions with the addition to the medium of 0.1 unit (approximately 5  $\gamma$ ) of insulin<sup>2</sup> per ml., except as specifically indicated in Tables II and III. Details of the procedure are described under Series II in a preceding paper (3).

Pituitary fractions were tested by intraperitoneal injection. The fractions were dissolved or suspended by grinding in 0.9 per cent sodium chloride immediately prior to injection. The solution of crystalline growth Fraction II was facilitated by the addition of 0.2 volume of 1 M tris(hydroxymethyl)aminomethane buffer at pH 8.0 (4). Control injections of solvents had no effect on the glucose uptake by diaphragm. Adrenal cortical extract<sup>3</sup> was injected intramuscularly. Appropriate controls were run with each set of injected animals.

*Preparation of Fractions from Anterior Pituitary*—Beef pituitaries were placed in dry ice shortly after removal from the animal and kept frozen until used, which was usually within 3 days of collection. The anterior lobes were removed in the frozen state, mixed with powdered dry ice, and put through a coffee grinder to give a coarse frozen powder. This was extracted overnight at 2° with calcium hydroxide at pH 11.5; ordinarily, 300 gm. of ground anterior lobe were extracted in a total volume of 2 liters. The resulting suspension was adjusted to pH 8.7 with carbon dioxide and left standing for several hours in the ice box. The undissolved material was removed by centrifugation and discarded. The various products described below were obtained by further fractionation of the supernatant fluid of pH 8.7 at 2°. Ten batches of pituitary were worked up over a period of 15 months, all of which yielded active fractions. In general, a single fraction was prepared from a given batch of glands.

*Crude Fraction I*—An aliquot of the supernatant fluid of pH 8.7 was dialyzed for several hours at 2° against distilled water and lyophilized. 1 mg. of the powder was obtained from 24 mg. of fresh anterior pituitary.

<sup>2</sup> Two types of insulin were employed, a Lilly commercial sample, 80 units per ml., and a Novo sample which was free of hyperglycemic factor.

<sup>3</sup> This was a commercial sample of Upjohn lipoadrenal cortical extract (Lot 52782TK), provided for this work through the courtesy of Dr. M. H. Kuizenga of The Upjohn Company.

*Crude Fraction II*—50 per cent<sup>4</sup> ethanol was added to the supernatant fluid of pH 8.7 until a concentration of 19 per cent was reached. The precipitate was collected by centrifugation, resuspended in distilled water, and lyophilized. 1 mg. of powder was obtained from 30 mg. of fresh glands.

*Crude Fraction III*—The supernatant fluid of pH 8.7 was dialyzed for several hours against distilled water at 2°. 50 per cent ethanol was added until a concentration of 13 per cent was reached. The precipitate was collected by centrifugation, resuspended in distilled water, and lyophilized. 1 mg. of powder was obtained from 52 mg. of fresh glands.

*Crystalline Growth Fraction I*—This was prepared according to the original method of Fishman, Wilhelmi, and Russell (5). The product, which has high growth activity, is partially crystalline but not electrophoretically homogeneous. Ethanol was added to the supernatant fluid of pH 8.7 until a concentration of 13 per cent was reached. The precipitate was collected by centrifugation and redissolved in calcium hydroxide at pH 11; this solution was adjusted to pH 8.6. The resulting precipitate was discarded and ethanol was added to the supernatant fluid to give a concentration of 7 per cent. The partly crystalline precipitate was collected by centrifugation, resuspended in distilled water, and lyophilized. 1 mg. of powder was obtained from 660 mg. of fresh glands.

*Crystalline Growth Fraction II*—This was prepared essentially according to the improved procedure of Wilhelmi, Fishman, and Russell (6), which yields a crystalline growth hormone preparation of greater homogeneity than crystalline growth Fraction I. A fresh batch of crude Fraction II was taken up in 0.1 M KCl and the pH adjusted to 11. The pH was then lowered to 5.0 and the precipitate discarded. The pH of the supernatant fluid was readjusted to 8.7, and ethanol was added until a concentration of 5 per cent was reached. The precipitate was discarded. The addition of ethanol was then continued to a final concentration of 20 per cent. The quality of the crystals obtained was improved by maintaining the pH at 7.9 by addition of an occasional drop of 1 N KOH. The crystals were collected by centrifugation, resuspended in distilled water, and lyophilized. For recrystallization, the crystals were dissolved in 0.1 M KCl and the above procedures repeated. 1 mg. of crystals can be obtained from about 350 mg. of fresh anterior pituitary (6), but the yield here obtained was somewhat less, as no effort was made to achieve maximum recovery of active material from discarded fractions.

### Results

All rates of glucose utilization are expressed as mg. per gm. of wet diaphragm per hour.

<sup>4</sup> All alcohol concentrations are expressed as volumes per cent.

*Effect of Hypophysectomy and Hypophysectomy Plus Adrenalectomy on Glucose Uptake by Diaphragm (Table I)*—The glucose uptake of the isolated diaphragm was measured in a series of forty-nine hypophysectomized rats. The rate of glucose utilization without added insulin was 3.8 and with added insulin 5.5 mg. The rates for normal diaphragm under the same conditions were 2.7 and 4.5 mg. These results confirm the earlier report that the glucose uptake by rat diaphragm is greatly increased during the period of 10 to 69 days after removal of the pituitary. When diaphragms are tested from rats 10 days or less after hypophysectomy, this increase may not be apparent (3, 7, 8).

TABLE I  
*Glucose Uptake by Diaphragm from Normal, Hypophysectomized, and Hypophysectomized-Adrenalectomized Sprague-Dawley Rats*

Rat preparation	Period of fasting	No. of rats	Glucose uptake, mg. per gm. wet tissue per hr.	
			In glucose	In glucose + insulin
	<i>hrs.</i>			
Normal . . . . .	20-24	19	2.7 $\pm$ 0.08*	4.5 $\pm$ 0.10
Hypophysectomized	20-24	49	3.8 $\pm$ 0.10	5.5 $\pm$ 0.10
Hypophysectomized-adrenalectomized	3-20	16	3.1 $\pm$ 0.09	5.9 $\pm$ 0.25

Test for significance of differences. Normal, no insulin, *versus* hypophysectomized, no insulin,  $P = <0.001$ . Normal, no insulin, *versus* hypophysectomized-adrenalectomized, no insulin,  $P = 0.003$ . Normal, with insulin, *versus* hypophysectomized or hypophysectomized-adrenalectomized, with insulin,  $P = <0.001$ .

\* Standard error.

Hypophysectomized rats were adrenalectomized and the glucose uptake of the diaphragm measured 48 to 72 hours later. These animals remained in good condition but became moribund with hypoglycemic convulsions when fasted 24 hours, a fasting period easily tolerated by hypophysectomized rats. The rate of glucose uptake in the absence of added insulin was intermediate between the values for the normal and the hypophysectomized animals.<sup>5</sup> In the presence of insulin the rate was not significantly different from that for the hypophysectomized series.

*Effect of Pituitary Extracts on Glucose Uptake by Diaphragms from Normal*

<sup>5</sup> Villee and Hastings (2) found the rate of glucose uptake by diaphragm from animals adrenalectomized and subsequently hypophysectomized to be higher than after hypophysectomy alone. In the experiments here reported the two glands were removed in the reverse order, and it should therefore be investigated whether this is responsible for the difference in the results obtained.

*and Hypophysectomized Rats*—In the non-fasted normal Anheuser-Busch rat large doses of crude Fraction II reduced the uptake of glucose from 3.0 to 1.6 mg., the level observed previously in diabetic preparations. Crystalline growth Fraction I at a dosage of 3 mg. per 100 gm. was equally effective. The addition of insulin increased the uptake significantly in both cases (Table II).<sup>6</sup>

In the hypophysectomized rat, both crude Fraction III at a dose of 10 mg. and crystalline growth Fraction I at a dose of 3 mg. produced a statistically significant reduction in the rate when injected 3 to 6 hours prior to the removal of the diaphragm. Neither crude Fraction I at a dose of 5 to 10 mg. nor crystalline growth Fraction II at a dose of 3 to 5 mg. was active

TABLE II

*Glucose Uptake by Diaphragm from Normal, Non-Fasted Anheuser-Busch Rats after Intraperitoneal Injection of Various Fractions of Anterior Pituitary*

Type of pituitary fraction injected <sup>a</sup>	Dose per 100 gm. rat	Time between injection and removal of diaphragm	No. of rats	Glucose uptake, mg. per gm. wet tissue per hr.	
				In glucose	In glucose + insulin <sup>†</sup>
	mg.	hrs.			
None, controls . . . . .			6	3.0 ± 0.23†	4.2 ± 0.38
Crude Fraction II . . . .	25-50	4	8	1.6 ± 0.17	2.8 ± 0.21
Crystalline growth Fraction I . . . . .	3	4	6	1.8 ± 0.11	3.5 ± 0.24

Tests for significance of differences. Control, no insulin, *versus* crude growth Fraction II, no insulin,  $P = 0.001$ . Control, no insulin, *versus* crystalline growth Fraction I, no insulin,  $P = 0.001$ .

<sup>a</sup> See the text for the preparation of the fractions.

<sup>†</sup> 0.01 unit of insulin per ml. was used in this series.

‡ Standard error.

under these circumstances, but both produced a depression in rate when injected 24 hours before the diaphragm was removed. At a single dose of 0.1 mg., which is approximately the total amount of growth hormone required over a 10 day period for optimal growth response (10), crystalline growth Fraction II was inactive. The decreased glucose uptake in muscle of hypophysectomized rats after injection of pituitary fractions was in each instance counteracted by the addition of insulin *in vitro* (Table III).

<sup>6</sup> Not included in Table II are experiments with a crude saline extract. Injection of the equivalent of 2 gm. of anterior pituitary 4 to 24 hours prior to the removal of the diaphragm did not depress glucose uptake. Similar results with this type of extract were obtained by Corkill and Nelson (9). The suppression of the effect of added insulin which they described has not been observed in our experiments.

**Role of Adrenals**—In hypophysectomized-adrenalectomized animals neither crude Fraction III alone nor adrenal extract alone produced a depression of glucose uptake by the diaphragm; with concurrent injections of both extracts there was a significant and substantial depression (Table IV).

TABLE III

*Glucose Uptake by Diaphragm from Hypophysectomized Rats (Anheuser-Busch and Sprague-Dawley Strains) after Intraperitoneal Injections of Various Fractions of Anterior Pituitary*

All animals were fasted 20 to 24 hours prior to removal of diaphragms.

Type of pituitary fraction injected*	Dose per 100 gm. rat	Time between injection and removal of diaphragm	No. of rats	Glucose uptake, mg. per gm. wet tissue per hr.	
				In glucose	In glucose + insulin
	mg.	hrs.			
None, controls			49	3.8 $\pm$ 0.10†	5.5 $\pm$ 0.10
Crude Fraction I	5 - 10	3- 6	5	3.6	5.0
	10	19-26	11	2.7 $\pm$ 0.16	4.9 $\pm$ 0.10
Crude Fraction III	10	3- 6	15	3.1 $\pm$ 0.14	5.3 $\pm$ 0.16
	10‡	4	9	2.1 $\pm$ 0.08	4.6 $\pm$ 0.14
Crystalline growth Fraction I	3	3	9	2.5 $\pm$ 0.16	3.8 $\pm$ 0.21§
Crystalline growth Fraction II	3 - 5	3- 4	9	3.5 $\pm$ 0.28	5.4 $\pm$ 0.13
	0.1	24	4	3.5	5.5
	3	24	8	2.8 $\pm$ 0.20	5.2 $\pm$ 0.13

Test for significance of differences Control, no insulin, *versus* crude growth Fraction I (19 to 26 hours), no insulin,  $P = <0.001$ . Control, no insulin, *versus* crude growth Fraction III, no insulin,  $P = 0.001$ . Control, no insulin, *versus* crystalline growth Fraction I, no insulin,  $P = <0.001$ . Control, no insulin, *versus* crystalline growth Fraction II at a dose of 3 mg. given 24 hours prior to test, no insulin,  $P = <0.001$ .

\* See the text for the preparation of the fractions.

† Standard error.

‡ These rats were also injected with adrenal cortical extract, 0.25 ml. each, at 24 and 4 hours prior to removal of diaphragm.

§ 0.01 unit of insulin per ml. was used in this series. The value of 3.8 is not significantly lower than the control value of 4.2 obtained concurrently at this insulin concentration.

In hypophysectomized animals, in which production of adrenal cortical steroids may be expected to be reduced, there was an augmentation of the action of crude Fraction III by the concurrent administration of adrenal cortical extract, as shown by a decrease in the rate of glucose uptake from 3.1 to 2.1 mg. (Table III).

These results show that adrenal cortical activity strongly enhances the

depressing effect of pituitary on glucose uptake by muscle, and may actually be necessary for the pituitary to become effective. This raises the question whether adrenotropic hormone was present in some of the growth hormone fractions used and whether stimulation of the adrenals by this highly active pituitary factor contributed to some of the observed effects. For example, the difference in activity between crystalline growth Fractions I and II (Table III) may have been due to the presence of different amounts of adrenotropic hormone. Until these points have been clarified, it is difficult

TABLE IV

*Glucose Uptake by Diaphragm from Hypophysectomized-Adrenalectomized Rats after Intraperitoneal Injection of Pituitary and Adrenal Cortical Extracts*

All rats were of the Sprague-Dawley strain and were fasted 4 to 6 hours prior to removal of the diaphragm. Pituitary extract was given at a dose of 10 mg. per 100 gm. of rat at 4 hours; adrenal cortical extract was given in two doses, 0.25 ml. each, at 24 and 4 hours prior to the use of the animals.

Injection*	No. of rats	Glucose uptake, mg per gm. wet tissue per hr.	
		In glucose	In glucose + insulin
Saline	16	3.1 $\pm$ 0.09†	5.9 $\pm$ 0.25
Crude Fraction III	10	3.2 $\pm$ 0.17	5.6 $\pm$ 0.14
Adrenal cortical extract	14	3.1 $\pm$ 0.14	6.1 $\pm$ 0.03
Crude Fraction III + adrenal cortical extract	10	2.4 $\pm$ 0.15	5.3 $\pm$ 0.15

Test for significance of differences. Saline, no insulin, *versus* crude Fraction III plus adrenal cortical extract, no insulin,  $P = 0.001$ . Adrenal cortical extract, with insulin, *versus* crude Fraction III plus adrenal cortical extract, with insulin,  $P < 0.001$ .

\* See the text for the preparation of the fractions.

† Standard error.

to say whether the active material is identical with the growth hormone or is a separate entity.<sup>7</sup>

<sup>7</sup> Since this paper was sent to press, a depression of the glucose uptake of diaphragms from hypophysectomized rats has been observed at the following doses of growth hormone, which are substantially below those recorded in Table III: (a) a single dose of 200  $\gamma$  of crystalline growth hormone (Armour, Lot 22KR2) per 100 gm. injected 24 hours prior to removal of the diaphragm; (b) eight daily doses of 40  $\gamma$  of crystalline growth Fraction II per 100 gm. given concurrently with 80  $\gamma$  per 100 gm. per day of a purified preparation of adrenocorticotrophic hormone; this dosage of adrenocorticotrophic hormone alone caused some restoration of adrenal weights but did not cause growth or exert a depressant effect on glucose uptake.

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#### SUMMARY

1. Various fractions of the anterior pituitary at dosages of 3 mg. or more per 100 gm., injected intraperitoneally 3 hours prior to removal of the diaphragm, had the following effects on the glucose uptake of the isolated muscle. In normal rats glucose uptake was depressed to the level observed previously in diabetic preparations; in hypophysectomized rats the increased rate of glucose uptake was returned to normal, and with concurrent injection of adrenal extract, it was depressed to a still lower level.

2. The glucose uptake by diaphragm from hypophysectomized-adrenalectomized rats, which was above normal, was *not* reduced by the injection of a pituitary fraction that was active in the rat lacking only the hypophysis. With the concurrent administration of adrenal extract, inactive by itself at the dosage level employed, the same pituitary extract produced a significant depression of glucose uptake.

3. Insulin brought about an increase in glucose uptake both in the absence and in the presence of pituitary-adrenal secretions.

4. The pituitary factor is stable to alcohol and to drying from the frozen state and follows the growth hormone during fractionation, including crystallization.

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# ON THE DETERMINATION OF LIVER XANTHINE OXIDASE AND THE RESPIRATION OF RAT LIVER HOMOGENATES\*

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The two general methods employed for estimating xanthine oxidase activity are (1) manometric measurement of the rate of oxygen consumption and (2) determination of the decolorization time of methylene blue in the presence of xanthine or hypoxanthine. Both methods have been used widely in studies of the purified enzyme (*e.g.* (1, 2)). Each has been adapted to the estimation of xanthine oxidase in liver, the manometric procedure by Axelrod and Elvehjem (3) and the methylene blue technique by Figge and Strong (4).

The purpose of the present paper is (1) to consider some factors affecting the manometric estimation of liver xanthine oxidase; (2) to compare the manometric and methylene blue techniques for the estimation of liver xanthine oxidase, and to show the correlation of the two methods as the xanthine oxidase activity of rat livers is varied under different dietary conditions (5); and (3) to provide additional evidence that at least 50 per cent of the *initial* endogenous respiration of rat liver homogenates is contributed by the oxidation of purine substrates.

## EXPERIMENTAL

**Warburg Procedure**—Liver xanthine oxidase was determined by the method of Axelrod and Elvehjem (3), but with Warburg manometers having 0.2 cc. of 20 per cent KOH in the center well. Urate oxidation was also determined simultaneously as described (3). Purified xanthine oxidase was prepared from milk by the method of Ball (1). Variations in activity of liver xanthine oxidase were obtained by dietary means (5).

In this determination the endogenous respiration of the liver homogenate was largely dissipated in a 40 minute incubation period prior to tipping in the xanthine. The xanthine oxidase activity was calculated from the excess  $O_2$  consumption in the xanthine flasks during the subsequent 20 minute reading periods; only those values that fell on the linear part of the curve were chosen. All manometric determinations were run in duplicate and the final values for xanthine oxidase activity were calculated as c.mm. of  $O_2$  consumed per gm. of dry liver per hour. 1 c.mm. of  $O_2$  per

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gm. of dry liver per hour was considered to be 1 unit of xanthine oxidase activity.

*Xanthine Oxidation by Liver Homogenates*—Typical oxygen consumption curves for three different livers of varying xanthine oxidase activity

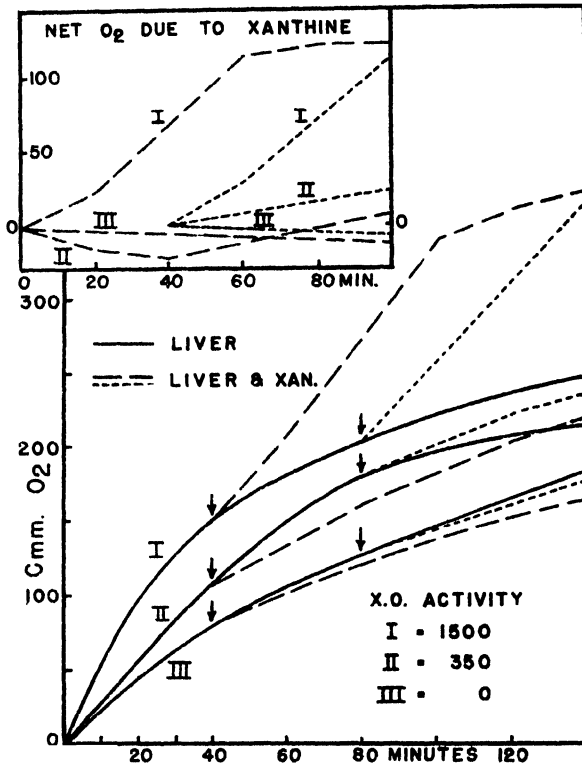


FIG. 1. Oxygen consumption curves for three different liver homogenates with xanthine oxidase activities of 1500, 350, and 0 c.mm. of O<sub>2</sub> per gm. of dry liver per hour. Solid line, endogenous respiration; dash line, oxygen consumption when xanthine was tipped in (arrow) after 40 minutes; dotted line, effect of tipping in xanthine after 80 minutes. Inset, excess oxygen consumption in the presence of xanthine.

are shown in Fig. 1. For the liver with a normal xanthine oxidase activity of 1500 units, the oxygen consumption followed the curves previously described by Axelrod and Elvehjem (3), and there was no difference in response when the xanthine was tipped in at 40 or 80 minutes after starting the measurement. However, when the xanthine oxidase activity was below approximately 500 units, tipping in xanthine at the usual 40 minute point produced an appreciable inhibition of the endogenous respiration;

so that the total oxygen consumption in the xanthine flasks never greatly exceeded that of the control flasks during the entire 100 minute run (Curve II, Fig. 1). It should be noted that the 20 minute increments of oxygen consumption in the xanthine flasks eventually exceeded the corresponding increment in the controls (inset, Fig. 1), and this excess allowed a calculation of 350 units for the xanthine oxidase activity of this particular liver, even though the total oxygen consumption in the xanthine flasks was less than in the controls. This calculation was justified by the results obtained when xanthine was tipped in after 80 minutes incubation (Curve II), whereupon the inhibition of the endogenous respiration was avoided, and the net oxygen consumption due to xanthine occurred at a rate equivalent to an activity of 350 units. In Curve III, Fig. 1, the oxygen consumption in the xanthine flasks was less than in the controls during each 20 minute period, and the xanthine oxidase activity of such livers could only be recorded as zero. A similar continuous but less intense inhibition of the endogenous respiration was observed when the addition of xanthine was delayed to the 80 minute point. Such "zero" livers have been shown by other means to contain small amounts (50 to 100 units) of xanthine oxidase.

This inhibition of the endogenous respiration upon addition of xanthine was observed in normal livers when the xanthine was tipped in after 10 minutes instead of after the usual 40 minute incubation. However, the oxygen consumption in the xanthine flasks eventually surpassed the controls at about the same time and to the same degree that would have resulted from adding the xanthine at the usual 40 minute point. These results with livers of both high and low activity varied in degree only, and indicated that (1) some portion of the endogenous substrate was preferentially oxidized before the xanthine oxidation became apparent, and (2) a more complete removal of this endogenous substrate prior to adding the xanthine prevented the inhibition effects. The relatively low oxygen consumption during the first 20 minute period after tipping in xanthine in a typical normal run is a further manifestation of this inhibition effect, since the conditions are theoretically optimal for a maximum rate of xanthine oxidation.

The inhibition by xanthine was not due to the alkali in which the xanthine was dissolved, since tipping in the same amount of alkali alone had no effect on the endogenous respiration. It was apparently unrelated to carbohydrate metabolism since (1) the addition of 1.5 to 30 mg. of glucose to the flasks at the start of the determination had no effect on the endogenous oxygen consumption or the pattern of the response to xanthine, and (2) fasting the rats for 24 hours to eliminate liver glycogen stores was similarly without pronounced effect. The average xanthine oxidase activity of five fasted rats was  $1380 \pm 58$  units, and both the endogenous

respiration and the response to added xanthine were similar to the results observed with fed rats.

The mechanism of xanthine inhibition appeared to be related to the known inhibition of xanthine oxidase by a relative excess of substrate (6). In Fig. 2 are oxygen consumption curves for two livers, with different xanthine oxidase activities, showing the effect of adding 0.15 cc. of 0.05 M hypoxanthine in 0.05 N NaOH to the flask at the start of the run. When

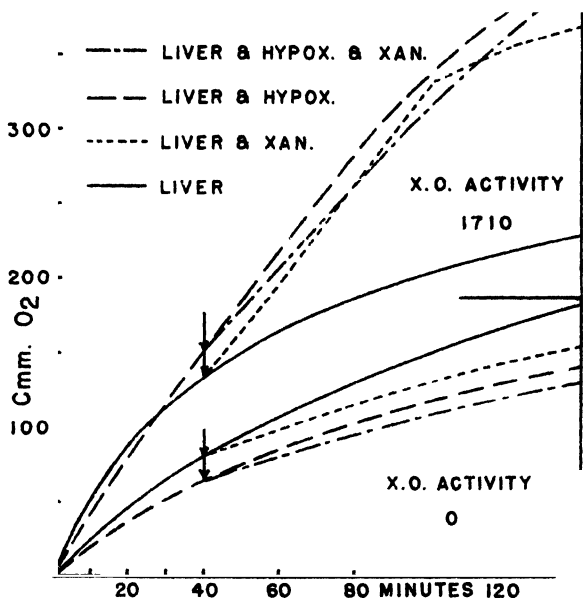


FIG. 2. The effect of added hypoxanthine on the oxygen consumption of two different liver homogenates with xanthine oxidase activities of 1710 and 0 c.mm. of O<sub>2</sub> per gm. of dry liver per hour. Solid line, endogenous respiration; xanthine tipped in at arrow to give the dotted line; dash line, oxygen consumption in the presence of hypoxanthine added to the body of the flask; xanthine tipped in at arrow to give the dash-dot line.

the xanthine oxidase activity was high, the hypoxanthine inhibited only slightly at first and gave a sustained high endogenous oxygen consumption; tipping in xanthine under such circumstances gave an inhibition of the respiration instead of the marked stimulation observed in the absence of added hypoxanthine. When the xanthine oxidase activity of the liver was zero, added hypoxanthine inhibited the endogenous respiration throughout the run, and tipping in the xanthine then gave a further inhibition.

Adding xanthine (0.15 cc. of 0.05 M) to the body of the flask at the start of the run and tipping in more xanthine at 40 minutes gave results similar to those shown for hypoxanthine in Fig. 2 for livers of both normal and

zero activity. Adding guanine to normal liver homogenate had little or no effect on the early respiration, but a high endogenous rate was continued for 60 to 80 minutes because of the oxidation of the guanine; addition of xanthine at 40 minutes gave slight or no inhibition of the oxygen consumption, but the true xanthine oxidase activity became measurable only after the guanine oxidation was complete. With livers of zero activity, added guanine inhibited the endogenous respiration slightly, and tipping in xanthine at 40 minutes gave a further slight inhibition.

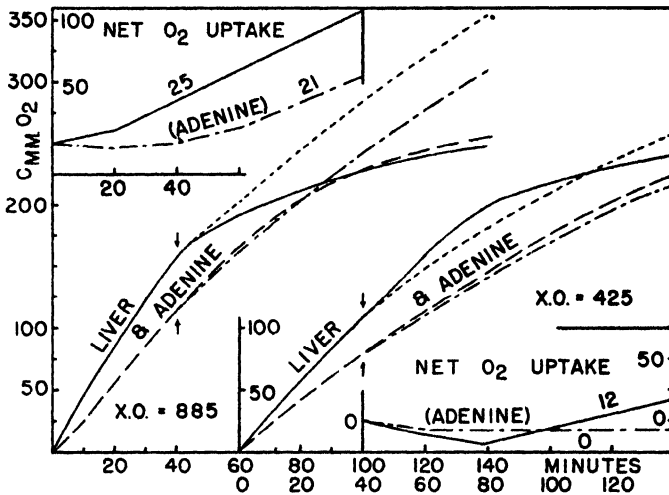


FIG. 3. The effect of added adenine on the oxygen consumption curves of two different liver homogenates. Solid line, endogenous respiration; xanthine tipped in at arrow to give the dotted line; dash line, oxygen consumption in the presence of adenine added to the body of the flask; xanthine tipped in at arrow to give the dash-dot line. Insets, excess oxygen consumption in the presence of xanthine. Solid line, no adenine added; dash-dot line, in presence of adenine. Figures along the curves refer to c.mm. of O<sub>2</sub> consumed per 20 minute period.

Adenine (0.15 cc. of 0.05 M) was not oxidized by normal rat liver homogenate, and, when added after an initial 40 minutes incubation, it had no effect on the oxygen consumption. When adenine was added to the body of the flask at the start of the run, it inhibited the early endogenous respiration, but the slower rate was sustained until the total oxygen consumption was nearly identical with that of the controls. Typical curves are shown in Fig. 3. Normal liver (1550 units) gave results similar to those shown for the liver with 885 units of xanthine oxidase activity; adenine inhibition of zero livers was similar to that shown in the curves for the 425 unit liver. In the presence of adenine the oxidation of xanthine was apparently slowed until the endogenous respiration had caught up

with the adenine-free controls; thereafter the oxidation of xanthine occurred at nearly the same rate in the presence or absence of adenine. Actually the apparent inhibition of xanthine oxidation in the presence of adenine was due to the sustained endogenous respiration rather than to a true inhibition of xanthine oxidation. With livers of low xanthine oxidase activity the endogenous respiration in the presence of adenine never caught up with the adenine-free controls and no xanthine oxidase activity could be observed. Without being oxidized itself, adenine apparently interfered with the removal of a substrate in the homogenate that was normally oxidized during the first 40 minutes, and the continued presence of it in samples of low xanthine oxidase activity interfered with the determination by giving a relatively sustained endogenous respiration and the xanthine inhibition effect. This endogenous substrate would seem to be a precursor of xanthine or hypoxanthine, since the endogenous oxygen consumption in the presence of adenine was continued at nearly

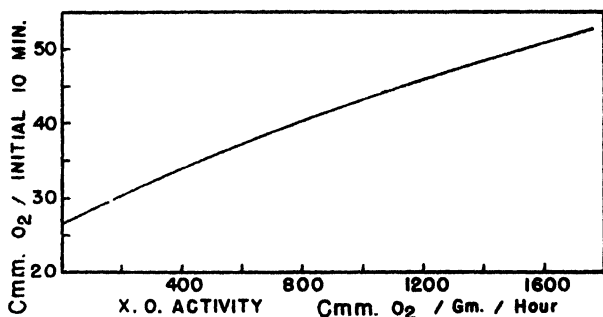


FIG. 4. The correlation of the endogenous respiration of liver homogenate during the first 10 minute measurement with the xanthine oxidase activity of the liver (average of 360 determinations).

the same rate during the later oxidation of xanthine. If endogenous xanthine or hypoxanthine were being oxidized by the homogenate at the time the xanthine was tipped in, no increased rate of oxygen consumption could be expected, and the xanthine oxidase activity under such circumstances would be "zero."

*Endogenous Rate*—The initial rate of the endogenous respiration paralleled the xanthine oxidase activity of the liver, as is shown in Fig. 4. When the xanthine oxidase activity of the liver was normal, the endogenous respiration during the first 10 minutes was approximately 50 c.mm. of O<sub>2</sub>. When the liver was nearly devoid of xanthine oxidase, the corresponding endogenous oxygen consumption was approximately 25 c.mm. of O<sub>2</sub>. While these figures are indicative of the altered respiration in the absence of xanthine oxidase, an exact comparison is unrealistic because of the appreciable endogenous respiration that is dissipated during the

10 minute equilibration period. The oxygen consumption of a normal liver homogenate during the first 10 minutes was approximately 80 c.mm. instead of 50 c.mm. when the equilibration period was reduced from 10 to 5 minutes.

When the xanthine oxidase activity was normal (1400 to 1800 units), the high initial endogenous rate dropped off rapidly after the first 40 minutes of incubation, the oxygen consumption per minute during the next 20 minutes being less than 35 per cent of that recorded initially. If a moderate endogenous respiration continued beyond the point at which the xanthine was added, the xanthine oxidase activity was usually found to be appreciably lower. When the endogenous oxygen consumption was initially low, it usually persisted with only a moderate falling off in rate throughout the major part of the 140 minute measurement. These differences can be noted in the curves in Fig. 1, and indicate that the major part of the endogenous substrates is oxidized during the first 40 minutes when the xanthine oxidase activity is normal, but that the endogenous respiration is slowed and the substrates persist when the xanthine oxidase activity is low.

*Urate Oxidation*—The oxidation of uric acid by normal liver homogenates, as described by Axelrod and Elvehjem (3), was nearly completed in 20 minutes. With livers of low xanthine oxidase activity the urate oxidation was only about 75 per cent complete at this point. However, when the urate addition was delayed until the endogenous respiration had proceeded for 40 minutes, normal oxidation of urate was observed, irrespective of the xanthine oxidase activity of the liver. Hence, liver uricase was unaffected by the dietary procedures, and variations in liver xanthine oxidase activity were not secondarily due to fluctuations in uricase. The apparent slight decrease in the uricase activity of livers with low xanthine oxidase activity can be explained as another manifestation of the inhibition produced by an added purine (7).

*Xanthine Oxidase Addition and Dialysis*—Liver homogenates of varying xanthine oxidase activity were studied before and after dialysis, with and without the addition of purified milk xanthine oxidase. For dialysis, the liver homogenate was prepared with 2 volumes of water, dialyzed 18 to 24 hours, and subsequently diluted with water and concentrated phosphate buffer to obtain the same proportions used in the undialyzed sample. All other details of the experiments were identical with those previously described; so that the results would be directly comparable.

In Fig. 5 are shown the results of a typical experiment in which purified xanthine oxidase was added to a liver homogenate of "zero" activity. The following points may be noted. (1) The addition of milk xanthine oxidase restored the endogenous respiration to that of a typically normal curve. (2) The inhibition of the endogenous respiration by the addition

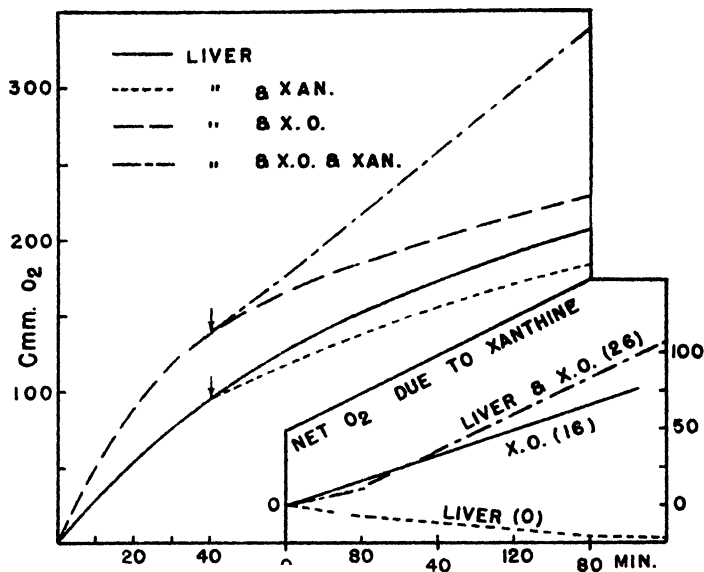


FIG. 5. The effect of adding purified xanthine oxidase on the oxygen consumption curve of a liver homogenate with a xanthine oxidase activity of zero. Solid line, endogenous respiration; xanthine tipped in at arrow to give the dotted line; dash line, oxygen consumption in the presence of xanthine oxidase added to the body of the flask; xanthine tipped in at arrow to give the dash-dot line. Inset, excess oxygen consumption in the presence of xanthine. Figures in parentheses refer to c.mm. of  $O_2$  per 20 minute period, the value for purified xanthine oxidase being taken only from the first 20 minute measurement.

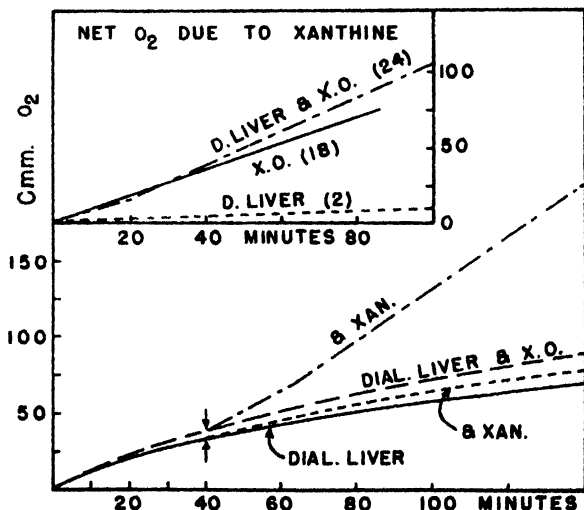


FIG. 6. The same as Fig. 5 except that the liver homogenate was dialyzed

of xanthine to livers of low xanthine oxidase activity was not observed in the presence of added xanthine oxidase. (3) An amount of purified xanthine oxidase which gave an oxygen uptake of 16 c.mm. per 20 minutes in an isolated system had a corresponding activity of 26 c.mm. in the presence of the liver homogenate; numerous other experiments also indicated that the enzyme activity was increased approximately 1.6 times in the presence of liver homogenate. Axelrod and Elvehjem (3) reported a ratio of 2:1. Boiled preparations of xanthine oxidase were without effect.

A portion of the same liver used above was homogenized and dialyzed against running tap water and finally distilled water for a total of 20 hours. The results of the usual type of Warburg experiment with the dialyzed homogenate are shown in Fig. 6. A small residual endogenous respiration was observed, probably owing to incomplete dialysis. This residual endogenous respiration was not inhibited by the addition of xanthine; hence the substrate whose oxidation was inhibited in the undialyzed homogenate was removed by dialysis. A very small but definite xanthine oxidase activity was noted in the dialyzed homogenate. This activity of approximately 2 c.mm. of  $O_2$  per 20 minutes (70 units) was observed consistently in nearly all livers whose activity before dialysis was recorded as zero because of the persisting inhibition produced by xanthine. The addition of milk xanthine oxidase to the dialyzed liver shows the presence of only small amounts of purine substrates remaining after dialysis. The activity of the purified xanthine oxidase was increased to the same extent as that found for undialyzed homogenate.

Studies similar to these were also made with livers showing intermediate and normal levels of xanthine oxidase activity. As the xanthine oxidase activity of the liver approached normal values, the addition of purified xanthine oxidase had less effect on the endogenous respiration, because smaller increases were required to give the normal curve. When added to livers of normal activity, the purified xanthine oxidase had what appeared to be a slight inhibiting effect on the endogenous respiration, as is shown in Fig. 7. This inhibition, however, proved to be an artifact, resulting from the more complete oxidation of free purine substrates in the liver homogenate during the initial 10 minute equilibration period; so that by the time the readings were started the liver homogenate containing added xanthine oxidase had less substrate available for oxidation, and the oxygen uptake was comparatively reduced. This was demonstrated by placing the purified xanthine oxidase in the side arm and tipping it into the liver homogenate at the time the readings were started. The endogenous respiration was then increased during the first 20 minutes instead of being decreased, but both the control and enzyme-



enriched preparations had consumed the same total amount of oxygen by 30 minutes and had similar respiration curves thereafter (Fig. 7). The nature of the response to added xanthine oxidase further shows that only a limited amount of free hypoxanthine or xanthine exists in fresh liver; however, there may have been a continuous formation of hypoxanthine or xanthine from some precursor during the incubation, which would not have been detected by these experiments.

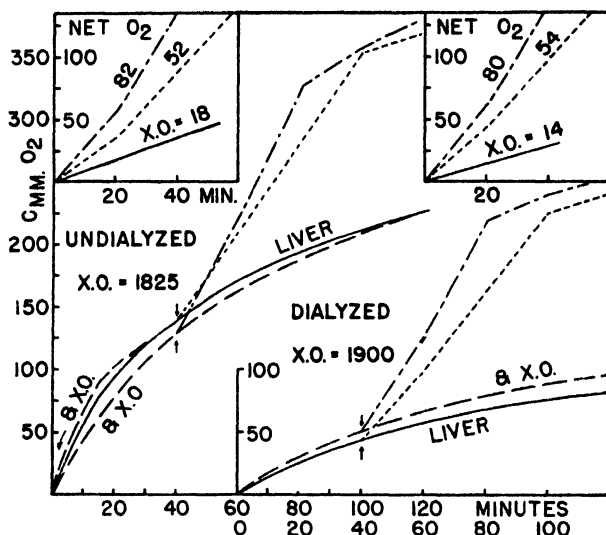


FIG. 7. The effect of adding purified xanthine oxidase on the oxygen consumption curve of a normal liver homogenate before and after dialysis. Curves at left, undialyzed; at right, dialyzed. Solid line, endogenous respiration; xanthine tipped in at arrow to give the dotted lines; dash line, oxygen consumption in the presence of xanthine oxidase added to the body of the flask; xanthine tipped in at arrow to give the dash-dot line. The effect of tipping in xanthine oxidase at zero time to an undialyzed preparation is also shown. Insets, excess oxygen consumption in the presence of xanthine. Figures along curves refer to c.mm. of O<sub>2</sub> per 20 minute period, the value for purified xanthine oxidase being taken only from the first 20 minute measurement.

The xanthine oxidase activity before and after dialysis was the same within approximately 10 per cent, for livers of both intermediate (700 units) and normal levels (1600 to 1800 units) of activity. Many of the livers showed slight losses of activity accompanying the dialysis. With both dialyzed and undialyzed livers the activity of added xanthine oxidase (12 to 18 c.mm. of O<sub>2</sub> per 20 minutes) was increased an average 1.6-fold and was then summated with the activity already present. Fig. 7 shows the effect of added xanthine oxidase on the same normal liver before and after dialysis. Boiled enzyme was without effect.

Livers of new-born rats, which had a zero xanthine oxidase activity without much if any inhibition of the endogenous respiration on addition of xanthine, responded to added xanthine oxidase (14 to 18 c.mm. of  $O_2$  per 20 minutes) by an increase in the endogenous curve to normal or nearly normal levels. However, the enzyme activity recovered in the homogenate approximately equaled that added instead of being increased some 1.6-fold, as was observed for more mature livers. After dialysis the livers of new-born rats still failed to show any xanthine oxidase activity.

*Methylene Blue Procedure*—A weighed sample of liver was homogenized (Potter) with 2 volumes of water and a few drops of toluene. A 6 cc. aliquot ( $\approx$  2 gm. of liver) was dialyzed in a cellophane bag against running tap water for 4 hours and then against a large volume of distilled water containing a few drops of toluene for 18 to 24 hours in a cold room; the bags were rocked continuously during the initial 4 hour dialysis. The contents of the bag were then removed as completely as possible and diluted to a convenient volume (usually 8 cc.). Each duplicate Thunberg tube contained 2 cc. of this dialyzed liver homogenate ( $\approx$  0.5 gm. of fresh liver) and 0.2 cc. of 0.5 M sodium phosphate buffer, pH 7.2. The side arm contained 0.1 cc. of 0.0113 M methylene blue and 0.2 cc. of 0.5 per cent hypoxanthine in 0.05 N NaOH. For the blank determination 0.2 cc. of distilled water replaced the hypoxanthine solution. The tubes were evacuated and flushed with nitrogen five times. After a 2 minute equilibration period in a water bath at  $38^\circ$ , the tubes were inverted, rapidly mixed, and replaced in the bath. The decolorization time from the point of mixing to the disappearance of the blue color was reproducible within 10 seconds in most determinations.

The blank decolorization time was usually between 20 and 30 minutes. All blanks could be extended to 50 minutes or more by an additional 16 hours dialysis. However, there seemed to be no particular advantage in a prolonged blank, since there was no correlation between the blank and the decolorization time observed with hypoxanthine.

Rat livers of varied xanthine oxidase activities were studied simultaneously by the Warburg and methylene blue techniques, and the results were plotted in a scatter diagram, Fig. 8. A decolorization time of 2 minutes in the procedure described was equivalent to a xanthine oxidase activity of 1800 c.mm. of  $O_2$  per gm. per hour in the Warburg procedure. A decolorization time of 5 minutes was equivalent to 750 c.mm. of  $O_2$  per gm. per hour. Between these limits there existed a straight line relationship between the two methods. With lower levels of enzyme activity the decolorization time was progressively prolonged (2). Testing such livers with one-half and one-fourth the usual amounts of hypoxanthine gave a significant decrease in decolorization time, but not enough to bring

the points to the theoretical straight line. Two livers whose xanthine oxidase activities were determined to be zero by the Warburg method (because of continued inhibition of the respiration by xanthine) gave decolorization times of 48 and 20 minutes, with corresponding blanks of 92 and 77 minutes. The presence of some enzyme in these livers was indicated by these results, but the amount was small.

The internal accuracy of the methylene blue procedure appeared to be less than that obtained by the Warburg method. Although duplicate Thunberg determinations on the same dialysate were consistently re-

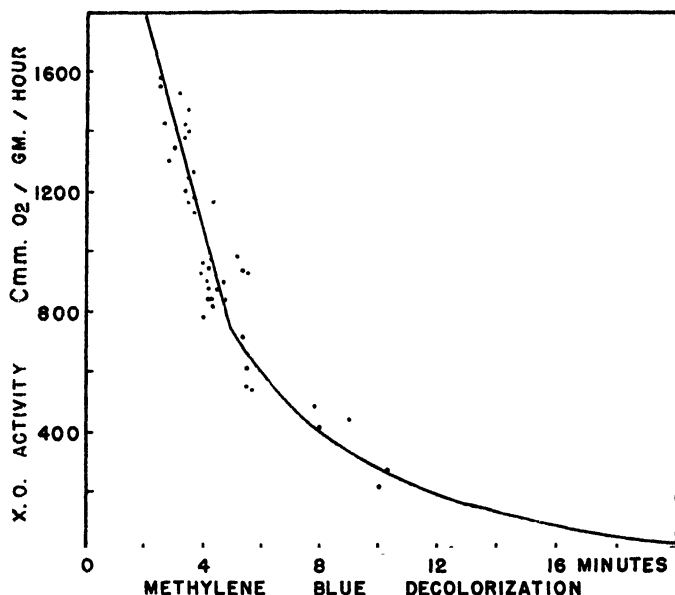


FIG. 8. Scatter diagram showing the relationship between the xanthine oxidase activity of liver measured by the manometric and methylene blue techniques.

producible, two independent determinations on the same homogenate dialyzed separately occasionally showed appreciable variation, which was not correlated with any variation in the decolorization time of the blanks. With the Warburg values as points of reference, the over-all error in the methylene blue procedure seldom exceeded 35 seconds, or 200 units (c.mm. of O<sub>2</sub> per gm. per hour).

A comparison of the methylene blue reduction and O<sub>2</sub> consumption on the same liver reveals that the limiting factor in the oxidation of hypoxanthine by xanthine oxidase is the reoxidation of the reduced enzyme rather than the removal of hydrogen from the substrate. In the methylene blue procedure hydrogen is removed from the hypoxanthine substrate

at a rate whose equivalent oxygen consumption can be calculated and shown to exceed the actual rate of oxygen consumption in the Warburg procedure.

The increased rate of reaction of the enzyme with hypoxanthine compared with xanthine in the methylene blue reduction (8) was not observed in the Warburg procedure. Both xanthine and hypoxanthine gave identical rates of oxygen consumption when tested with purified milk xanthine oxidase. When added to liver in a typical xanthine oxidase determination, the rate of oxidation of hypoxanthine was 80 to 100 per cent of the rate obtained with xanthine. This would be expected if reoxidation of the enzyme were the limiting factor in the aerobic oxidation of both xanthine and hypoxanthine.

*Methylene Blue Aerobically*—It is possible to increase the rate of oxygen consumption by adding methylene blue as a hydrogen carrier to the aerobic system (9). The addition of 0.15 cc. of 0.0113 M methylene blue to the Warburg flask increased the activity of purified milk xanthine oxidase (16 to 22 c.mm. of  $O_2$  per 20 minutes) about 3-fold with xanthine as the substrate; with hypoxanthine substrate the increase was initially 2-fold, but increased to 3-fold after the first 10 minutes. When added to liver homogenates, the methylene blue increased the xanthine oxidase activity 1.2- to 2.0-fold as the xanthine oxidase activity of the liver varied from 2300 to 350 units. When the methylene blue was added to livers whose xanthine oxidase activities were zero because of continued inhibition of the endogenous respiration by the xanthine, some activity was consistently detected, usually in the region of 200 units. Hence, the true xanthine oxidase activity of such zero livers in the absence of methylene blue was about 100 units. This would agree well with the 70 units found after dialysis.

The effect of methylene blue on the endogenous oxygen consumption of liver homogenate varied with the xanthine oxidase activity of the preparation and with the time of addition of the methylene blue; the results were similar to but more marked than those obtained with added xanthine oxidase. Typical curves are shown in Fig. 9. When added to a liver homogenate of low xanthine oxidase activity (350 to 720 units), the methylene blue increased the early endogenous rate, but the rate then fell off to less than the control values. When added to normal liver (1900 units) before measurements were started, the oxygen consumption was appreciably less than the control values throughout the run, owing to the more rapid and complete exhaustion of substrates in the homogenate during the equilibration period. Tipping in methylene blue at the time the measurements were started resulted in an increased oxygen consumption for the first 10 minutes, with a subsequent falling off to about the

control level and a paralleling of the control curve thereafter. Tipping in methylene blue after 40 minutes of incubation had no effect on the endogenous curve.

*Inhibitors*—Sodium arsenite in a final concentration of 0.005 M inhibited the endogenous respiration of rat liver appreciably, and decreased the xanthine oxidase activity to about one-fifth of the normal levels obtained in the absence of added arsenite. Arsenite was previously reported to inhibit the xanthine oxidase in skin (10).

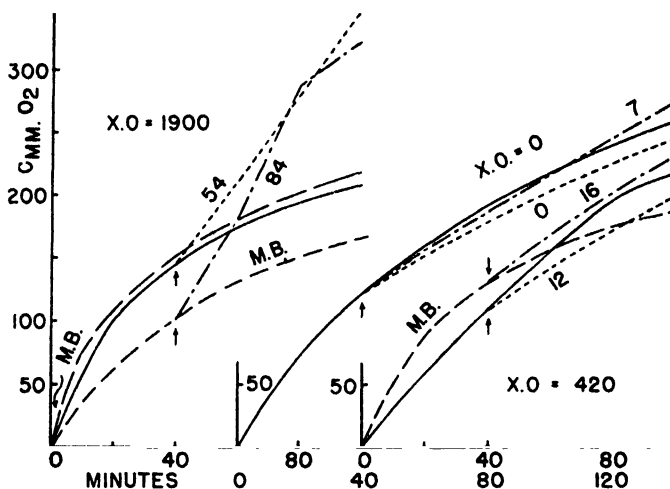


FIG. 9. The effect of methylene blue on the oxygen consumption curves of three liver homogenates of varying xanthine oxidase activities. Solid line, endogenous respiration; xanthine tipped in at arrow to give the dotted line; dash line, oxygen consumption in the presence of methylene blue added to the body of the flask; xanthine tipped in at arrow to give the dash-dot line. The effect of tipping in methylene blue at zero time is also shown for the liver with a xanthine oxidase activity of 1900. Adding methylene blue to the liver with zero xanthine oxidase activity did not change the endogenous respiration appreciably. Numbers along curves refer to the net oxygen consumption due to xanthine oxidation in c.m.m. per 20 minutes.

Iodoacetate in a final concentration of 0.01 M was reported (11) to have no appreciable effect on milk xanthine oxidase or liver aldehyde oxidase in the methylene blue procedure. The effect of iodoacetate on the xanthine oxidase activity of rat liver was found to depend upon the time of addition. When iodoacetate was tipped in with the xanthine or hypoxanthine substrate after the usual 40 minutes incubation, the xanthine oxidase activity was decreased by only 15 to 30 per cent. However, when the iodoacetate was added to the homogenate before readings were begun (at least 70 minutes before making the actual measurements), the xanthine oxidase

activity of the liver with either xanthine or hypoxanthine substrate was reduced to one-fourth or one-fifth of the value obtained in the absence of iodoacetate (1600 to 2000 units); the endogenous respiration was inhibited very little. The lack of effect of iodoacetate on the endogenous respiration can be explained by the time lag preceding the inactivation of xanthine oxidase.

Bernheim *et al.* (12, 13) reported that the quinimine form of *p*-aminophenol was a relatively specific inhibitor of milk and liver xanthine oxidase. In our experiments the addition of oxidized *p*-aminophenol to give a final concentration of 0.0002 M inhibited the endogenous respiration of normal liver (1800 units) during the first 40 minutes by only 20 to 30 per cent, while it completely inhibited the oxidation of xanthine when the latter was tipped in at 40 minutes. However, when the oxidized *p*-aminophenol and xanthine were tipped in together at either the 10 or 40 minute point, there was no inhibition of the xanthine oxidation. Similarly, if the xanthine were added to the body of the flask and oxidized *p*-aminophenol were tipped in at the 10 minute reading, the oxidation of xanthine was inhibited only slightly. Under the conditions of these experiments xanthine protected the enzyme from the inhibition effects of oxidized *p*-aminophenol, and the relatively small effect of this inhibitor on the early endogenous respiration may be attributed to the protection of the enzyme by the oxidation of endogenous purine substrates.

#### DISCUSSION

The results are consistent with the following interpretation. In a normal rat liver homogenate at least 50 per cent of the *initial* oxygen consumption is due to the oxidation of purine substrates (14); only a limited amount of free xanthine or hypoxanthine is present in the homogenate initially, but additional amounts are formed from some unidentified precursor during the incubation. The major portion of these purine substrates is oxidized within the first 40 minutes when the xanthine oxidase activity is normal. This results in a high endogenous rate of oxygen consumption, no accumulation of purines in the homogenate at the time xanthine is added, and a relatively straight forward oxidation of the added xanthine. When the xanthine oxidase activity of the liver homogenate is low, the endogenous purine substrates are oxidized more slowly, and the slower endogenous rate tends to be sustained for a longer period of time, because the decreased enzyme activity would require a longer time to remove a given amount of endogenous purine substrates. At the time the xanthine is usually tipped in, the homogenate still contains endogenous purine substrates in the process of being oxidized, and the added xanthine inhibits this oxidation by providing a relative excess of substrate for the limited amount of enzyme

present (6). The oxidation of the added xanthine can be observed only after the endogenous purines are exhausted, and the rapidity with which this occurs depends upon the xanthine oxidase activity of the original liver homogenate.

Theoretically the manometric estimation of liver xanthine oxidase could give falsely low values because of a persisting oxidation of endogenous purine substrates, and this has been observed with the "zero" livers that actually contain small amounts of xanthine oxidase. At least six instances have also been encountered in over 1000 determinations in which a high endogenous respiration has continued throughout the entire 140 minute run, and in which the determined xanthine oxidase activity was lower than that expected from the endogenous rate and appreciably lower than the averages of livers from the other rats in the same dietary groups. Such determinations are suspect. Fortunately they are rare, and the good correlation between the manometric, and methylene blue procedures supports the view that the manometric procedure is generally reliable. Independent but simultaneous manometric determinations on the same liver gave values that agreed within 70 units (2 c.mm. of  $O_2$  per 20 minutes) in most cases.

Freshly prepared solutions of xanthine were oxidized by the liver homogenate less rapidly than solutions that had aged several months, and correspondingly gave about 10 per cent lower xanthine oxidase activity for the liver. This effect may be related to the finding (15) that irradiation of the solutions to produce small amounts of peroxide hastened the methylene blue reduction by the xanthine oxidase system.

#### SUMMARY

The initial endogenous rate of oxygen consumption by rat liver homogenate was proportional to the xanthine oxidase activity of the sample. Low endogenous rates tended to persist and were inhibited by the addition of xanthine. Adding xanthine to a homogenate actively oxidizing xanthine or hypoxanthine inhibited the rate of oxygen consumption. Adenine slowed and prolonged the early endogenous respiration and prevented the determination of xanthine oxidase activity until the accumulated endogenous substrates had been oxidized. Urate oxidation was normal in livers of varying xanthine oxidase activity.

The activity of liver xanthine oxidase was essentially unchanged by dialysis. Small amounts of xanthine oxidase were found in "zero" livers after the removal of endogenous substrates by dialysis.

The addition of purified milk xanthine oxidase to liver homogenate of low activity restored the endogenous respiration to normal and prevented the xanthine inhibition effect. With both dialyzed and undialyzed liver

the activity of added milk xanthine oxidase was increased on an average 1.6-fold and was then summated with the activity already present.

A methylene blue reduction procedure for liver xanthine oxidase was described and correlated with the manometric method.

Since the enzyme was reduced by the substrate faster than it was re-oxidized by air, the addition of methylene blue to the aerobic system increased the activity of milk and liver xanthine oxidase 3- and 1.6-fold respectively. Methylene blue affected the endogenous oxygen consumption in the same way as did added xanthine oxidase; it also showed the presence of small amounts of xanthine oxidase in "zero" livers.

Liver xanthine oxidase was inhibited by arsenite, iodoacetate, and oxidized *p*-aminophenol; the iodoacetate inhibition was delayed. The enzyme was protected from the inhibiting effects of oxidized *p*-aminophenol during the active oxidation of xanthine.

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## STUDIES ON OLIGOPHRENIA PHENYLPYRUVICA

### I. MICROBIOLOGICAL DETERMINATION OF L- AND D-PHENYLALANINE AND OF PHENYLACTIC ACID\*

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The disease oligophrenia phenylpyruvica (phenylketonuria) presents a unique opportunity for the study of the relation of amino acid metabolism to the function of the central nervous system. In this inborn error of metabolism, which is inherited as a Mendelian recessive character, mental deficiency is accompanied by faulty phenylalanine metabolism. The metabolic disturbance was first recognized by the urinary excretion of large amounts of phenylpyruvic acid (3). It was soon recognized, however, that the voiding of the keto acid is accompanied by an abnormally high excretion of phenylalanine and phenyllactic acid as well. The source of the excreted amino acid and its metabolic derivatives is probably the high concentration of phenylalanine in the blood of these subjects.

Two explanations have been proposed for the biochemical mechanism of the metabolic error. Fölling and his collaborators suggested the metabolic accumulation of D-phenylalanine and its subsequent deamination in the kidney as the source of the excreted keto acid, but they were unable to demonstrate the presence of the unnatural antipode in body fluids (4). Jervis has presented strong evidence that the metabolic disturbance resides in an inability to hydroxylate the aromatic ring (5). At present it is not known whether the inability to hydroxylate is complete, because of the total lack of a specific enzyme system, or whether only parahydroxylation is affected. The impaired ability of many patients with this disease to form melanin points to a more extensive disturbance of the metabolism of the aromatic nucleus (6). Furthermore, the lack of quantitative data on the occurrence of the various metabolic products makes a correlation between the degree of mental impairment and the extent of the metabolic deficiency impossible.

Microbiological methods, because of their sensitivity and specificity, seemed to offer a valuable tool for the study of this metabolic disturbance. We report here a method for the microbiological determination of each isomer of phenylalanine and of DL-phenyllactic acid and its application to

\* Aided by grants from the Rockefeller Foundation and the New York Foundation. Preliminary reports have appeared elsewhere (1, 2).

the assay of these compounds in body fluids. The method is based on the finding that *Lactobacillus arabinosus* can use either isomer of phenyllactic acid in the place of L-phenylalanine for growth, but is unable to utilize D-phenylalanine. The natural amino acid is determined in one aliquot of an unknown solution and the total phenylalanine is determined in another aliquot after conversion of the amino acid to phenyllactic acid by treatment with nitrous acid. The difference between the results of the two analyses is equivalent to the amount of unnatural isomer present. Phenylpyruvic acid was also found to replace L-phenylalanine in the metabolism of *L. arabinosus*; therefore both the hydroxy and the keto acid, if present, must be removed first by chemical methods if the amino acid alone is to be determined.

#### EXPERIMENTAL

**Bacteriological Assay**—In agreement with others (7) we have found that there is growth of *L. arabinosus* on a phenylalanine-free medium during an incubation period of 72 hours, but, as we reported previously (1), during a 40 to 44 hour incubation period there is no visible growth in the absence of phenylalanine. The medium of Hac, Snell, and Williams (8), with phenylalanine omitted, was employed. The incubations were carried out for 40 hours in 25 × 100 mm. test-tubes, loosely plugged with cotton. The total volume was 2.5 ml. The growth, as determined turbidimetrically, was proportional to the L-phenylalanine concentration up to 8  $\gamma$  per ml. of culture medium.

DL-Phenylalanine yielded one-half the bacterial growth of a corresponding weight of L-phenylalanine;<sup>1</sup> no growth was obtained on pure D-phenylalanine. Either D- or L-, or DL-phenyllactic acid replaced the L-amino acid in the same concentration (Table I).

Although phenylpyruvic acid can replace phenylalanine in the bacterial diet, growth is not proportional to the concentration of the keto acid and tends to be erratic, owing probably to the instability of the compound. In large amounts, above 120  $\gamma$  per ml., it actually inhibits bacterial growth during an incubation of 40 hours.

**Determination of Phenylalanine, Phenyllactic Acid, and Phenylpyruvic Acid in Presence of Each Other**—A solution containing from 0.1 to 1 mg. per ml. of each of the three compounds was used for the determination. The keto acid may be determined in an aliquot of the solution as its 2,4-dinitrophenylhydrazone by a modification of the Neuberg-Case procedure, but because of the non-specificity of the method, the values obtained in body fluids merely indicate the total keto acid content.

For the determination of phenylalanine and of phenyllactic acid the keto

<sup>1</sup> We are indebted to Dr. William H. Stein of the Rockefeller Institute for a sample of pure L-phenylalanine.

acid was destroyed by heating 1 ml. of the solution at 105° for 15 hours with 0.3 ml. of 6 N HCl in a sealed tube of 10 ml. capacity and 8 mm. inside diameter. A higher concentration of HCl was avoided, since the excessive amounts of sodium chloride formed on subsequent neutralization inhibit bacterial growth. The solution was transferred quantitatively to a 10 ml. volumetric flask and diluted to the mark (Solution A). A 3 ml. aliquot was neutralized to pH 7 and diluted to 10 ml. Appropriate aliquots of this solution were used for the microbiological assay of combined phenylalanine and phenyllactic acid. " Since the growth effect of the amino and hydroxy acids is additive, and since their molecular weights differ only by 0.6 per cent, the values for the sum of the two compounds may be read from the phenylalanine standard curve.

TABLE I  
*Growth of Lactobacillus arabinosus on Phenylalanine and Phenyllactic Acid*

Metabolite	Amount added	Growth in 40 hrs., optical density
	$\gamma$	
DL-Phenylalanine . . . . .	14	0.15
L-Phenylalanine . . . . .	2.5	0.06
" . . . . .	5	0.11
" . . . . .	7	0.15
" . . . . .	10	0.21
D-Phenylalanine . . . . .	14	0
DL-Phenyllactic acid . . . . .	2.5	0.06
" " . . . . .	5	0.11
" " . . . . .	7	0.15
D-Phenyllactic " . . . . .	7	0.15

For the determination of phenylalanine and phenyllactic acid separately, a 6 ml. aliquot of Solution A was extracted in a continuous extractor with ether for 6 hours or with chloroform for 1½ hours. The aqueous phase was quantitatively transferred to an Erlenmeyer flask, evaporated to a small volume on a hot-plate, neutralized to pH 7, and diluted to 10 ml. (Solution B). Phenylalanine was determined in an aliquot of this solution. The phenyllactic acid concentration may be calculated either as the difference between the values for combined phenylalanine-phenyllactic acid and for phenylalanine, or it may be determined directly in the ether extract (Table II).

*Determination of D-Phenylalanine*—1 ml. of a solution containing 50 to 100  $\gamma$  of a mixture of D- and L-phenylalanine was transferred to a 10 ml. volumetric flask and 0.5 ml. of a 4 per cent solution of NaNO<sub>2</sub> and 0.4 ml. of 1 N HCl solution were added. The flask was stoppered and gently

swirled for 20 seconds. Vigorous shaking of the flask led to low recoveries caused by the loss of nitrous acid. After an hour, another 0.5 ml. of  $\text{NaNO}_2$  and 0.4 ml. of  $\text{HCl}$  were added and the flask was again swirled for 20 seconds. After the solution had remained at room temperature for 18 hours, 1 ml. of 2 per cent urea solution was added. After 15 minutes the solution was neutralized to pH 7 and diluted to 10 ml. Aliquots of from 0.5 to 1.25 ml. were added to 1.25 ml. of the concentrated culture medium, and enough water to make a total volume of 2.5 ml. The solution was autoclaved, inoculated, and incubated for 40 hours. The value obtained represents the sum of both antipodes of phenylalanine. The difference between this value and that obtained from an identical aliquot without treatment

TABLE II  
*Determination of Phenylalanine (Ph-A) and Phenyllactic Acid (Ph-L) in Presence of Phenylpyruvic Acid (Ph-P)*

Experiment	Amount	Sum of (2) + (3)	Found after destruction of Ph-P					
			Before extraction, Ph-A + Ph-L		Ether extract, Ph-L		Residue, Ph-A	
	$\gamma$	$\gamma$	$\gamma$	per cent	$\gamma$	per cent	$\gamma$	per cent
(1) Ph-P	40							
(2) Ph-A	20							
(3) Ph-L	31	51	50	98	29	94	21	105
(1) Ph-P	7.5							
(2) Ph-A	15							
(3) Ph-L	11.7	26.7	27.2	102	12	103	14.9	99
C (1) Ph-P	11							
(2) Ph-A	22.5							
(3) Ph-L	17.6	40.1	40.1	100	18	102	22.5	100

with nitrous acid (L-phenylalanine) corresponds to the D-phenylalanine present. Since these determinations of L- and total phenylalanine were carried out on acid-hydrolyzed ultrafiltrates, the nitrous acid treatment converted all phenylalanine whether present originally as the free amino acid or bound in peptide linkage.

For the determination of D-phenylalanine in the presence of phenyllactic acid and phenylpyruvic acid, 1 ml. of Solution B was taken. In Table III the results of the determination of various proportions of D- and L-phenylalanine are shown. The recovery of L-phenylalanine is within 5 per cent and the recovery of the sum of D- and L-phenylalanine within 10 per cent of the amounts present. Since this deviation tends to be positive, a parallel standard curve with known amounts of D- and L-phenylalanine should be established, especially if the relative amount of the D isomer is low. To test the effect of other amino acids on the determination of D-phenyl-

alanine, DL-phenylalanine was treated with nitrous acid in the presence of basal medium (8) containing a 6-fold excess of all the known amino acids over the phenylalanine. The recovery of D-phenylalanine corresponded to that obtained with DL-phenylalanine alone.

*Determination of Phenylalanine and Its Metabolic Derivatives in Body Fluids*—Tungstic acid filtrates of plasma may be employed for the determination of phenylalanine but not of phenyllactic acid, which, it was found, is almost completely adsorbed on the protein precipitate. For this reason all determinations were performed on ultrafiltrates of plasma or serum. The ultrafiltrations were carried out under 30 pounds pressure at 4° for 16 hours with cellophane membrane No. 600.

TABLE III

*Determination of D-Phenylalanine in Presence of L-Phenylalanine*

Total phenylalanine analyzed, 7  $\gamma$ .

Phenylalanine		Bacterial growth*			Total phenylalanine found
D isomer	L isomer	Calculated	Found		
			Before HNO <sub>2</sub> treatment	After HNO <sub>2</sub> treatment	
per cent	per cent				γ
0	100		0.14	0.14	7
10	90	0.13	0.12	0.15	7.5
20	80	0.11	0.11	0.15	7.5
30	70	0.099	0.098	0.16	7.7
40	60	0.085	0.086	0.15	7.5
50	50	0.071	0.069	0.15	7.2

\* Optical density obtained from a standard curve based on L-phenylalanine for amounts of L-phenylalanine present in the mixture.

# RESULTS AND DISCUSSION

The conditions under which *L. arabinosus* is incubated determine whether this organism can grow without phenylalanine in its medium (7). On prolonged incubation in tightly stoppered tubes, from which the escape of carbon dioxide is prevented, it is able to synthesize phenylalanine readily. If the conditions of the incubation are well standardized, a reproducible growth response to DL-phenylalanine is obtained. Under the conditions described, the standard curves, determined weekly, have been the same in this laboratory for the past 2 years.

During studies of the dependence of bacterial growth on the length of incubation in a phenylalanine-free medium, it was noted that phenylalanine apparently inhibits growth on prolonged incubation (Table IV). During a 40 hour incubation there is no visible growth on a phenylalanine-free

medium and the growth response is proportional to the L-phenylalanine added. Between 40 and 72 hours there is rapid growth without phenylalanine, but no significant increment in those tubes which contain phenylalanine. It is noteworthy that bacterial growth is stabilized between 40 and 72 hours on a level proportional to the presence of added L-phenylalanine but that without phenylalanine growth exceeds that obtained with

TABLE IV  
*Inhibition of Bacterial Growth by Phenylalanine during Prolonged Incubation*

Substance added	Amount added per 2.5 ml.	Growth in 40 hrs., optical density	Growth in 72 hrs., optical density
	$\gamma$		
None . . . . .		0.03-0.04	0.18-0.25
D-L-Phenylalanine. . . . .	150	0.54	0.56
“ . . . . .	10	0.15	0.16
L-Phenylalanine. . . . .	70	0.51	0.53
“ . . . . .	5		0.16
“ . . . . .	2.5	0.10	0.11
D-Phenylalanine . . . . .	2.5	0.03	0.28
D-L-Phenyllactic . . . . .	4	0.13	0.14
Dopa. . . . .	2.5		0.24
“ . . . . .	5		0.27
“ . . . . .	10		0.29
“ + D-L-phenylalanine . . . . .	10 + 10		0.15

TABLE V  
*Concentration of D- and L-Phenylalanine in Serum of Patients with  
Oligophrenia Phenylpyruvica*

Patient	L-Phenylalanine per 100 ml.	Total phenylalanine per 100 ml.	D-Phenylalanine
	mg.	mg.	mg.
Ma. . . . .	34	34	0
R. . . . .	36	35	0
F . . . . .	35	34	0
Mu. . . . .	33	32	0

low levels of phenylalanine. The shorter incubation period is essential for assay purposes in order to secure a reproducible blank. During an incubation of 72 hours, wider variations in growth are obtained without phenylalanine than in the presence of the amino acid. These findings are reported here, since they have a bearing on the conditions of the assay of phenylalanine. The mechanism of this inhibition is being investigated.

The determination of D-phenylalanine was made possible by the ability of *L. arabinosus* to utilize both antipodes of the hydroxy acid but only the

L form of the amino acid. The method cannot be used in the determination of D-glutamic acid, for *L. arabinosus* cannot utilize hydroxyglutaric acid, but it may well be applicable to other amino acids.

L-Phenylalanine was determined in the protein-free blood plasma filtrates of seventeen normal subjects. In these the mean concentration was  $0.95 \pm 0.06^2$  mg. per cent; the total spread of the values was from 0.6 to 1.3 mg. per cent. These results are in agreement with those of other investigators (9). The method described above was applied to the determination of L- and total phenylalanine in the plasma of subjects with oligophrenia phenylpyruvica. The results (Table V) show that there was no measurable increment in bacterial growth after the conversion of the amino acid to the hydroxy acid, and that, therefore, no D-phenylalanine was present within the limit of error of the procedure. This finding makes it appear unlikely that the metabolic error in oligophrenia phenylpyruvica involves the formation of the unnatural isomer of phenylalanine.

#### SUMMARY

A microbiological method is described for the determination of D- and L-phenylalanine and of DL-phenyllactic acid in the presence of each other and in the presence of phenylpyruvic acid.

Phenylalanine was determined by this method in the blood plasma of normal subjects and of patients with oligophrenia phenylpyruvica.

No D-phenylalanine could be detected in the plasma of the mental defectives.

We are indebted to Dr. G. A. Jervis for his cooperation in securing blood samples from patients.

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<sup>2</sup> Standard error.





# ON THE METABOLISM OF PHENYLALANINE AND TYROSINE

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The metabolism of phenylalanine and tyrosine has been the subject of investigation for many years. Much evidence has been produced to show that in mammalian tissue phenylalanine is converted to tyrosine (1-5). Tyrosine can be oxidized to *p*-hydroxyphenylpyruvic acid, which can in turn form homogentisic acid (5-13). Embden, Salomon, and Schmidt (14) showed that phenylalanine, tyrosine, and homogentisic acid yield acetoacetic acid when perfused through a surviving liver. It has long been known that these amino acids have ketogenic activity *in vivo* (15-17). Recently, Winnick, Friedberg, and Greenberg (18) and Weinhouse and Millington (19) working with C<sup>14</sup>- $\beta$ -labeled DL- and L-tyrosine, respectively, showed that tyrosine is at least in part converted to acetoacetic acid in the rat and in rat liver slices. Radioactive acetoacetic acid was isolated after feeding the rat  $\beta$ -labeled DL-tyrosine and after incubating rat liver slices with  $\beta$ -labeled L-tyrosine. Schepartz and Gurin (20) incubated C<sup>14</sup> ring-labeled DL-phenylalanine with rat liver slices and showed for the first time that carbon atoms from the benzene ring are incorporated into acetoacetic acid. These workers also proved that if homogentisic acid is formed from phenylalanine the carbon side chain must shift on the benzene ring.

Weinhouse and Millington (19) and Schepartz and Gurin (20) concluded from the unequal distribution of isotopic carbon in the acetyl and acetate fractions of acetoacetate that ketone bodies are formed as intact 4-carbon units in the metabolism of phenylalanine and tyrosine. We considered it desirable to obtain additional evidence for this belief and to determine the nature of substances other than ketone bodies which might arise from the metabolism of the aromatic nucleus of these amino acids. To this end labeled optically active phenylalanine was synthesized with C<sup>14</sup> in the benzene ring and C<sup>13</sup> in the  $\alpha$ -carbon atom. Labeled, optically active tyrosine with C<sup>14</sup> in the  $\beta$  position was also used. The results of a study of the metabolism of these labeled amino acids in rat liver slices, summarized in Diagram 1, indicate that phenylalanine and tyrosine are metabolized to ketone bodies and malic acid (or its precursor). The experimental evidence for these findings is given in this paper.

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*Synthesis of Labeled Phenylalanine and Tyrosine*

The steps in the synthesis of phenylalanine labeled with  $C^{14}$  in the benzene ring and  $C^{13}$  in the  $\alpha$  position are given in Diagram 2.

**Hippuric Acid**—5.20 gm. of  $C^{13}$ -labeled methyl iodide were converted to  $C^{13}$ -methyl-labeled sodium acetate in 90 per cent yield through a nitrile synthesis and alkaline hydrolysis.<sup>1</sup> 3.5 gm. of the labeled sodium acetate were fused and then treated with bromine and phosphorus pentabromide to give bromoacetyl bromide (21). Water (in 10 per cent excess) was added to the bromoacetyl bromide reaction mixture to give monobromoacetic acid. The solution was treated with ammonia and benzoyl chloride

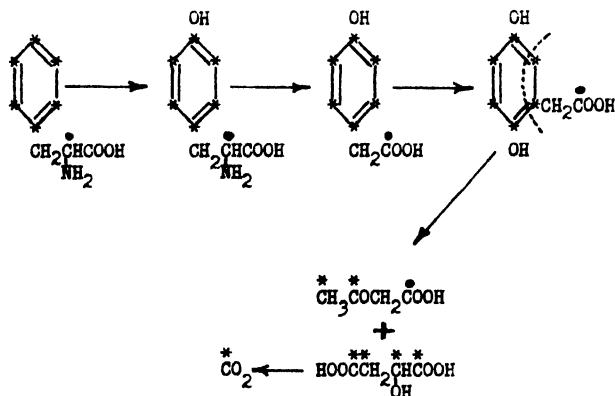


DIAGRAM 1. Metabolism of  $C^{14}$  ring-labeled,  $C^{13}$   $\alpha$ -labeled phenylalanine. \* denotes  $C^{14}$ ;  $\dot{C}$  denotes  $C^{13}$ .

(22) to give hippuric acid. 3.5 gm. (46 per cent of theory) of hippuric acid were obtained.

**Benzaldehyde**—Benzene was converted to benzaldehyde through a Gattermann reaction as described by Reformatsky (23). 3.187 gm. of benzene (Tracerlab) labeled with  $C^{14}$  in a single position and having a total activity of 0.5 mc. were diluted with 10 ml. of anhydrous cyclohexane in a 50 ml. centrifuge tube. The tube was cooled and kept in an ice bath throughout the entire reaction. 0.6 gm. of anhydrous cuprous chloride and 11.5 gm. of anhydrous aluminum bromide were added to the reaction. The mixture was stirred vigorously, and the anhydrous gases carbon monoxide and hydrogen chloride (in a 2:1 ratio) (24-25) were bubbled through the mixture for 30 minutes. The contents of the reaction tube were then slowly poured onto chips of ice. Benzaldehyde was extracted from the aqueous layer

<sup>1</sup> J. D. Bordeaux, to be published.

twice with 10 ml. portions of a benzene-cyclohexane (1:3) solution. The organic phase was shaken with an excess of alcoholic sodium bisulfite (26) and the resulting benzaldehyde-bisulfite complex was removed by filtration through a sintered glass funnel. The precipitate was washed with benzene and air-dried. 3.4 gm. (40 per cent of theory) of benzaldehyde-bisulfite

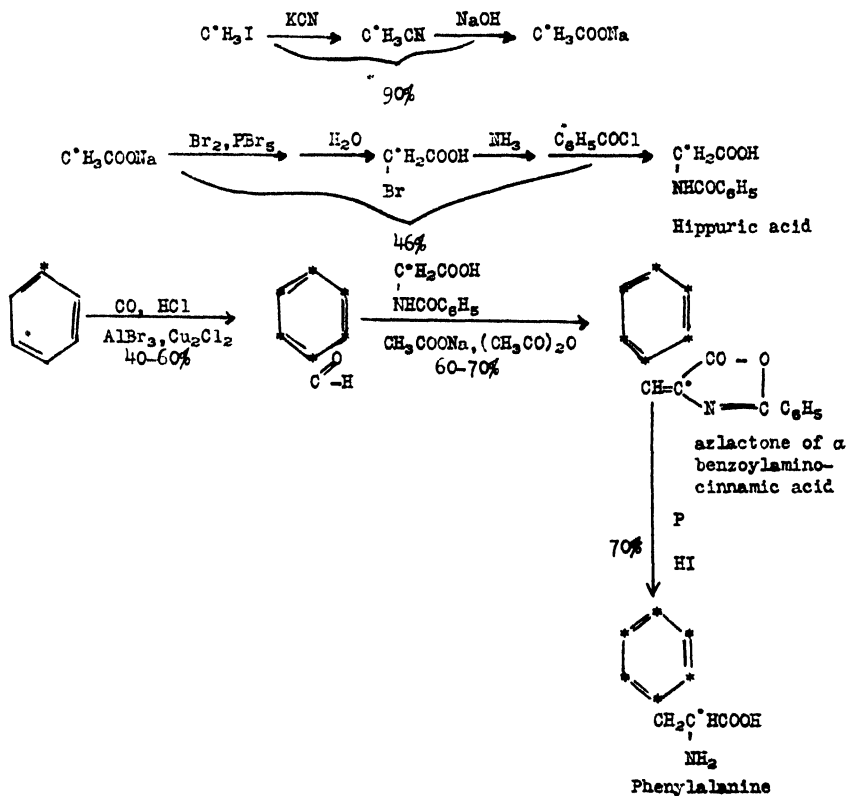


DIAGRAM 2. Synthesis of  $\text{C}^{14}$  ring-labeled,  $\text{C}^{13}$   $\alpha$ -labeled phenylalanine.  $\text{C}^*$  denotes  $\text{C}^{14}$ ;  $\text{C}$  denotes  $\text{C}^{13}$ .

complex were obtained. Control runs with non-isotopic benzene usually gave 60 per cent of theory.

The benzene-cyclohexane layer, after removal of the alcoholic bisulfite layer, was dried over anhydrous sodium sulfate and distilled. The fraction distilling at  $70^\circ$ , which contained some unreactive radioactive benzene, was reworked to give an additional amount of benzaldehyde of lower activity. The two benzaldehyde preparations were converted to phenylalanine separately.

*Azlactone of  $\alpha$ -Benzoylamino-cinnamic Acid*—3.4 gm. of benzaldehyde-bisulfite complex, 2.91 gm. of hippuric acid, 1.35 gm. of freshly fused sodium acetate, 9.2 ml. of acetic anhydride, and 1.0 ml. of acetic acid were added to 20 ml. of anhydrous benzene. The mixture was refluxed for 6 hours. The azlactone of  $\alpha$ -benzoylamino-cinnamic acid which crystallized on cooling was filtered and washed with a few ml. of boiling water. 2.19 gm. of azlactone were obtained.

*Phenylalanine*—The azlactone was treated with red phosphorus and hydriodic acid in the usual manner (27) and gave 0.760 gm. of pure DL-phenylalanine. The yield of phenylalanine was 11.3 per cent based on the initial amount of benzene and 11.7 per cent based on the initial amount of methyl iodide.

Resolution of the DL-phenylalanine to the optically active forms was carried out by the procedure of du Vigneaud and Meyer (28), in which the brucine salts of the formyl derivatives of phenylalanine are separated. After two recrystallizations from water and alcohol, 86.0 mg. of pure L-phenylalanine and 185.0 mg. of pure D-phenylalanine were obtained. Both preparations of phenylalanine decomposed at 284–288° and gave the expected amount of carbon dioxide when oxidized with chromic acid. The specific rotations were not determined on the isotopic phenylalanine preparations, but control non-isotopic preparations made in the same manner with the same reagents were resolved completely. Incubation of the radioactive L-phenylalanine with D-amino acid oxidase<sup>2</sup> prepared from sheep kidney showed no oxygen uptake, thus indicating that the L isomer of phenylalanine was not contaminated with the D isomer. The phenylalanine preparations had an activity of  $3.30 \times 10^6$  counts per minute per mm of phenylalanine and contained 9.45 per cent C<sup>13</sup> in the  $\alpha$  position.

*Tyrosine*—5.0 mg. of C<sup>14</sup>- $\beta$ -labeled DL-tyrosine<sup>3</sup> and 27.5 mg. of pure non-isotopic L-tyrosine were dissolved in 7.5 ml. of boiling water. On cooling, L-tyrosine precipitated and was separated from the mixture by filtration through a sintered glass funnel. The precipitate was washed with a few drops of cold water and dried at 110° for 1 hour. 14.1 mg. of L-tyrosine were obtained.

The filtrate was evaporated to dryness and 18.5 mg. of tyrosine were obtained. This fraction of tyrosine contained a mixture of D-tyrosine, having a high specific activity ( $47.8 \times 10^6$  counts per minute per mm), and L-tyrosine having a lower specific activity. To determine the efficiency of the resolution procedure, the radioactivity in each of the two preparations was determined. The L-tyrosine preparation had an activity of  $4.26 \times 10^6$

<sup>2</sup> I am indebted to Dr. E. Kearney and Dr. T. P. Singer for the purified D-amino acid oxidase preparation.

<sup>3</sup> Obtained through the kindness of Dr. Melvin Calvin, University of California.

counts per minute per mm of tyrosine and the mixed tyrosine fraction (filtrate residue) contained  $9.65 \times 10^6$  counts of tyrosine. The expected activity of L-tyrosine is  $3.98 \times 10^6$  counts per minute per mm of tyrosine (determined by calculating the total activity present in the 32.5 mg. of tyrosine from the above values). The value of  $3.98 \times 10^6$  is in good agreement with that of  $4.26 \times 10^6$  actually found and indicates that practically pure L-tyrosine was obtained.

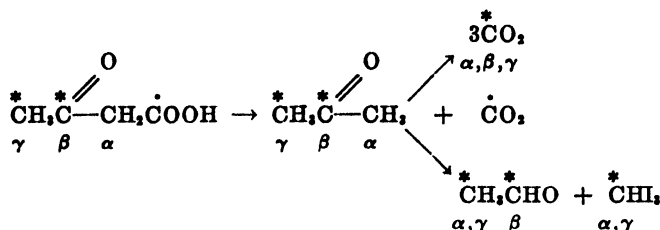
### *Experiments in Vitro and Results*

Liver slices from adult, male albino rats were suspended in Krebs' calcium-free 0.01 M phosphate buffer at pH 7.4 (29) with the appropriate substrate and shaken for 2 hours at 38°. Oxygen was passed over the reaction mixture continuously, and the exit gases were passed through a bubbler with 2.5 N sodium hydroxide to collect the respiratory CO<sub>2</sub>.

*Formation of Acetoacetic Acid*—The procedure followed in most of the experiments is exemplified by the following description. 2.0 mg. of C<sup>14</sup> ring-labeled L-phenylalanine were incubated with approximately 10 gm. (wet weight) of rat liver slices in 20 ml. of phosphate buffer as described above. After 2 hours the reaction was stopped. 35.7 mg. of respiratory carbon dioxide were found in the alkali. 2.5 ml. of an aqueous solution containing 105.1 mg. of acetoacetic acid were added as carrier. The mixture was diluted with 30 ml. of water and 20 ml. of 25 per cent cupric sulfate solution. Sufficient solid calcium hydroxide was mixed into the solution to make the mixture alkaline to litmus. After standing at room temperature for 45 minutes, the mixture was centrifuged at 3000 R.P.M. for 10 minutes and then filtered. 39 ml. of filtrate were obtained, which was brought to pH 2 with concentrated sulfuric acid. To remove any radioactive carbon dioxide present in the solution tank carbon dioxide and then nitrogen were bubbled through the solution at a rapid rate for periods of 5 minutes. Acetoacetic acid was converted to acetone and carbon dioxide according to the method of Van Slyke (30) as follows: After adding 35 ml. of 10 per cent mercuric sulfate and 10 ml. of 50 per cent sulfuric acid, the mixture was refluxed for 45 minutes. The acetone formed precipitated as Denigès' mercury complex, and the carbon dioxide derived from the carboxyl group was collected in a bubbler containing 3 ml. of 2.5 N sodium hydroxide. 18.1 mg. of carbon dioxide were obtained. The mercury-acetone complex was filtered off, washed with cold water, and dried at 110° for 1 hour. 487.4 mg. of mercury-acetone complex representing approximately 24.4 mg. of acetone were obtained. This value is in accord with that expected when 18.1 mg. of carbon dioxide come from the carboxyl group of acetoacetic acid.

The mercury-acetone complex was divided into two portions. One por-

tion (121.5 mg.) was oxidized quantitatively with chromic acid (31) to carbon dioxide. The second portion, representing 356 mg. of the mercury-acetone complex, was dissolved in 15 ml. of 6 N hydrochloric acid and the acetone was distilled directly into a cooled mixture of 30 ml. of 0.1 N iodine and 15 ml. of 17 N sodium hydroxide. The cooled receiving flask was shaken continuously during the distillation of acetone. By this procedure acetone was degraded to acetaldehyde and iodoform. The  $C^{14}$  activity of iodoform represents the mean activity of the two methyl groups of acetone. The iodoform, after being allowed to stand overnight, was filtered onto an asbestos filter and then dried over calcium chloride for 48 hours in a small desiccator. In this manner 59.9 mg. of iodoform (50 per cent of theory) were obtained. The iodoform was oxidized to carbon dioxide (4.7 mg., 70 per cent of theory) with chromic acid.<sup>4</sup> A brief résumé of the degradation of acetoacetic acid is as follows:



The various carbon dioxide fractions obtained from acetoacetate by the above procedures are carboxyl as  $\text{CO}_2$ ,  $\alpha$ ,  $\beta$ , and  $\gamma$  as acetone, and an average of  $\alpha$  and  $\gamma$  as iodoform. The  $C^{14}$  activity was determined with a Geiger counter on barium carbonate precipitated and filtered on paper disks. Control experiments with 2.0 mg. of non-isotopic L-phenylalanine yielded less than 1 mg. of acetoacetic acid. Since this amount is small compared with the total weight of carrier added in the above experiment (105.1 mg.), the activities calculated for 105.1 mg. of acetoacetic acid very nearly represent the total of isotopic acetoacetic acid present in the reaction mixture at the end of the experiment.

Results of the above experiment are shown in Table I and are in accord with the composition of acetoacetate as given in Diagram 1. It can be seen that the carboxyl group of acetoacetic acid has relatively little activity and that the remaining 3-carbon unit has much activity. If the acetoacetic acid is formed from phenylalanine, as illustrated in Diagram 1, it would be expected that all the radioactivity would be in the  $\beta$ - and  $\gamma$ -carbons and none in the  $\alpha$ - and carboxyl carbons. Calculation of the specific activity

<sup>4</sup> This particular batch of chromic acid was prepared from concentrated sulfuric acid instead of fuming sulfuric acid. Oxidation of iodoform with this preparation gives a 70 per cent yield of carbon dioxide.

of the  $\beta$ - and  $\gamma$ -carbons from the specific activities of the acetone gives 161.8 ( $107.9 \times 3/2$ ) and from the iodoform 160.8 ( $80.4 \times 2/1$ ). These results are thus in agreement with the proposed scheme. Experimental evidence supporting this view is found in the work of Weinhouse and Millington (19) who showed that acetoacetic acid was produced with  $C^{14}$  only in the  $\alpha$ -carbon atom when  $C^{14}$ - $\beta$ -labeled L-tyrosine was incubated with liver slices. The radioactive phenylalanine used in our experiments had no activity in the  $\beta$ -carbon atom, and hence no activity would be expected in the  $\alpha$ -carbon atom of acetoacetic acid. Since there is good evidence that in the liver phenylalanine is converted to tyrosine, the ketone bodies produced from phenylalanine and from tyrosine should arise by identical mechanisms. The present results support this view.

TABLE I  
*Distribution of  $C^{14}$  Activity in Acetoacetic Acid*

2.0 mg. of  $C^{14}$  ring-labeled L-phenylalanine ( $3.30 \times 10^6$  counts per minute per mm) were incubated with approximately 10 gm. of rat liver slices for 2 hours at pH 7.4 and 38°. Inactive acetoacetic acid carrier (105 mg.) was added to the system at the end of the reaction.

Carbon atoms in acetoacetic acid	Specific activity, counts per min. per mg. C	Total $C^{14}$ activity, counts per min.
Carboxyl . . . . .	19.6	242
$\alpha$ -, $\beta$ -, $\gamma$ . . . . .	107.9	4040
Average of $\alpha$ - and $\gamma$ . . . . .	80.4	1980

The question arises as to how  $C^{14}$  activity, even though relatively small, was found for the carboxyl group of acetoacetate. It will be seen shortly that radioactive malate is formed in the metabolism of ring-labeled phenylalanine. This means that small amounts of carboxyl-labeled acetoacetate could arise from 2-carbon units derived from the metabolism of labeled malate. Another explanation for finding some activity for the carboxyl group of acetoacetate is that it represents contamination from other readily decarboxylated radioactive substances (*e.g.* oxalacetate) that may be present in the reaction mixture. Activity in the carboxyl group does not appear to result from a randomization produced by cleavage of acetoacetate to acetate and then resynthesis of acetoacetate. It will be shown that the carboxyl group had much  $C^{13}$  activity, while the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -carbon atoms had very little  $C^{13}$  activity, thus indicating little randomization.

If ketone bodies ( $\beta$ -hydroxybutyric and acetoacetic acids) are derived from phenylalanine as intact 4-carbon units, the  $C^{13}$  and  $C^{14}$  of the phenylalanine should be diluted equally on conversion to acetoacetic acid, and the labeling should not be randomized in the acetoacetate. Since in the



experiments described above the excess  $C^{13}$  found in the carboxyl group of acetoacetic acid was insufficient for accurate measurement, similar experiments were carried out in triplicate, and the reaction mixtures from the three flasks were mixed with a total of 39.5 mg. of acetoacetic acid added as carrier. In this way dilution was reduced. The mixture was degraded as described above.

The results are shown in Table II. Carbon dioxide from the carboxyl group was found to contain 0.11 atom per cent excess  $C^{13}$ . The original phenylalanine had 9.45 atom per cent excess  $C^{13}$  in the  $\alpha$ -carbon atom. Hence, there was a 1:85.9 dilution of the  $C^{13}$  from the original phenylalanine to the acetoacetic acid which was isolated with the carrier.

$C^{14}$  activity of the acetone fragment of acetoacetic acid was determined after combustion of the mercury-acetone complex to carbon dioxide.

TABLE II

*Comparison of  $C^{14}$  and  $C^{13}$  Activities of Phenylalanine and Acetoacetic Acid*

6.0 mg. of  $C^{14}$  ring-labeled L-phenylalanine ( $3.30 \times 10^6$  counts per minute per mm) were incubated with approximately 30 gm. of rat liver slices for 2 hours at pH 7.4 and  $38^\circ$ . Inactive acetoacetic acid carrier (39.5 mg.) was added to the system at the end of the reaction.

Compound	$C^{14}$ activity of labeled carbon atoms, counts per min. per mg. C		$C^{13}$ activity of labeled carbon atom, per cent excess	
	Ring carbons		$\alpha$ -Carbon	
Phenylalanine		45,918	Carboxyl	9.45
Acetoacetic acid	$\beta$ - and $\gamma$ -	613.8		0.11
Dilution		74.8		85.9

Assuming that  $C^{14}$  activity is present in only 2 of the 3 carbon atoms of acetone, *i.e.* only the  $\beta$ - and  $\gamma$ -carbons of acetoacetic acid, it was found that the labeled carbon atoms had 613.8 counts per minute per mg. of carbon. The benzene carbons of the initial phenylalanine had 45,918 counts per minute per mg. of carbon. Hence,  $C^{14}$  activity was diluted 74.8 times, and this dilution is identical, within experimental limits, with the dilution of  $C^{13}$  activity. Only a trace (less than 0.02 atom per cent excess) of  $C^{13}$  was found in the carbon dioxide from the acetone fragment. These findings indicate that the 4 carbon atoms of acetoacetic acid are split off as a unit when phenylalanine is metabolized to ketone bodies.

*4-Carbon Unit from Benzene Ring*—Relatively little has been reported on the fate of the 4 ring carbon atoms of phenylalanine and tyrosine which are not involved in ketone body formation. It has been shown that these amino acids promote formation of liver glycogen in the fasting rat (32, 33). This suggests that the glycogen may be derived from this part of the molecule. The only evidence opposed to this view is that phlorhizinized ani-

mals excrete ketone bodies and no glucose when given phenylalanine or tyrosine (15). Actually, these data do not offer conclusive evidence against the formation of glycogenic substances, because such substances could be rapidly metabolized, whereas ketone bodies, which are metabolized relatively slowly, could be excreted in the urine.

In all of the experiments described above much  $C^{14}$  activity but no  $C^{13}$  activity was found in the respiratory carbon dioxide. It must be pointed out, however, that little  $C^{13}$  activity would be detectable because of the great dilution. These results indicated that carbon dioxide was formed from the ring carbons that were not involved in ketone body formation. Additional evidence for this belief is given by experiments described later in the paper, which show that little activity is present in respiratory carbon dioxide but much activity in ketone bodies when  $C^{14}$ - $\beta$ -labeled tyrosine is metabolized by liver slices. The results from the acetoacetic acid derived from  $C^{14}$ - and  $C^{13}$ -labeled phenylalanine indicated that 2 carbons of the benzene ring (plus 2 carbons of the aliphatic side chain) form ketone bodies. The question naturally arises as to how the 4 remaining carbon atoms of the ring are separated from the rest of the molecule. It is unlikely that 2-carbon fragments are formed, because if such a reaction occurred ketone bodies formed from the 2-carbon units would be labeled in all 4 carbons. This was not found to be the case. The formation of a single carbon unit, *e.g.* carbon dioxide, also is unlikely because the total activity in the respiratory carbon dioxide was always appreciably less than the total activity in the ketone bodies. This indicates that the formation of carbon dioxide is an indirect process and that many of its radioactive precursors remain in solution. It was, therefore, logical to assume that a 4-carbon unit (such as fumarate or malate) is formed which eventually is oxidized to carbon dioxide. Several years ago Neubauer (34) suggested that fumaric acid might be formed from phenylalanine and tyrosine. Since malate and fumarate are in equilibrium with each other in the presence of liver tissue and since this equilibrium is greatly in favor of malate, malic acid was used to test our hypothesis.

Experiments were set up as described above and 300 mg. of malic acid were added as carrier at the beginning of the reaction. Sufficient dilute sodium hydroxide was added to adjust the pH to 7.4. After the reaction had proceeded for 2 hours, 2.5 ml. of concentrated sulfuric acid were added and the entire reaction mixture was mixed with 100 gm. of calcium sulfate. Organic acids were separated from the calcium sulfate-tissue mixture by extraction with ether.<sup>5</sup> The ether was evaporated and 10 ml. of cold water were added. Insoluble fats were removed by filtration and the filtrate neutralized with a known quantity of dilute sodium hydroxide. The solu-

<sup>5</sup> J. Meyer, to be published.

tion was evaporated to dryness, and 50 per cent sulfuric acid (in 5 per cent excess of the sodium hydroxide previously used) was added to the residue. Approximately 100 mg. of anhydrous sodium sulfate and two portions of 3 ml. of solvent mixture (70 per cent chloroform and 30 per cent butanol saturated with 0.5 N sulfuric acid) were added. The two dry extracts were passed through a 15 gm. column of silica gel. After approximately 150 ml. of solvent had run through the column, the malic acid fraction appeared. Approximately 70 ml. of additional solvent were used to elute the malic acid. 69.6 mg. of malic acid were extracted from chloroform-butanol with water. By neutralizing the malic acid with sodium hydroxide and evaporating the solution to dryness, solid sodium malate was obtained.

The two carboxyl groups of malic acid were converted quantitatively to carbon dioxide by means of permanganate oxidation (35). In this oxida-

TABLE III  
*Distribution of C<sup>14</sup> Activity in Malic Acid*

2.0 mg. of C<sup>14</sup> ring-labeled L-phenylalanine ( $3.30 \times 10^6$  counts per minute per mm) were metabolized by approximately 10 gm. of rat liver slices for 2 hours at pH 7.4 and 38°. Inactive malic acid carrier (300 mg.) was added to the system at the beginning of the reaction. 69.6 mg. of malic acid were isolated at the end of the experiment.

Carbon atoms in malic acid	C <sup>14</sup> activity, counts per min. per mg. C
Carboxyl	154.7
$\alpha$ - and $\beta$ -	149.2

tion the 2 internal carbon atoms appeared as acetaldehyde, which was collected in a bisulfite bubbler. The theoretical amount of acetaldehyde was obtained. The acetaldehyde was oxidized quantitatively to carbon dioxide with persulfate (36).

The results presented in Table III show that radioactive malic acid is formed from phenylalanine and that the activity is equally distributed between the carboxyl and internal carbon atoms. This finding is consistent with the formation of a 4-carbon unit from the benzene ring.

The results presented in Table IV indicate that when ring-labeled L-phenylalanine is the substrate much activity is found in malic acid, but that when  $\beta$ -labeled L-tyrosine is the substrate almost no activity is found. 21.5 per cent of the activity of 4 ring carbon atoms from phenylalanine appeared as carbon dioxide or malic acid. This value is probably low because malic acid was not extracted quantitatively from anhydrous sodium sulfate prior to chromatographic analysis. From this observation one can

conclude that the formation of malic acid represents a major metabolic pathway in the oxidation of L-phenylalanine and tyrosine.

*Yield of Ketone Bodies*—If phenylalanine and tyrosine are metabolized to two 4-carbon fragments, one being malic acid (or its precursor) and the other a ketone body, the amount of ketone bodies produced from these amino acids should be comparable to the amount of malic acid produced. This was found to be the case.

Quantitative measurements of the total amount of radioactivity in acetoacetic acid and  $\beta$ -hydroxybutyric acid were carried out as follows: Separate experiments with 2.0 mg. of L-phenylalanine and 2.0 mg. of L-tyrosine were carried out in the usual manner. After acetoacetic acid was decarboxylated

TABLE IV

*Conversion of Phenylalanine and Tyrosine to Malic Acid and Carbon Dioxide*

2.0 mg. of  $C^{14}$  ring-labeled L-phenylalanine ( $3.30 \times 10^6$  counts per minute per mm) and 2.0 mg. of  $C^{14}$ - $\beta$ -labeled L-tyrosine ( $4.26 \times 10^6$  counts) were incubated with approximately 10 gm. of rat liver slices for 2 hours at pH 7.4 and  $38^\circ$ . Inactive malic acid carrier (300 mg.) was added to the system at the beginning of the reaction. At the end of the phenylalanine and tyrosine experiments 69.6 and 66.6 mg. of malic acid were isolated, respectively.

Initial substrate	Substance isolated at end of reaction	Specific activity, counts per min. per mg. C	Per cent of original activity found in final product*
Phenylalanine	Respiratory $CO_2$	165.0	7.3
	Malic acid	152.2	14.2
Tyrosine	Respiratory $CO_2$	7.5	0.5
	Malic acid	<4.0	0

\* See the text for the methods used to calculate these values.

and the mercury-acetone complex separated from the solution, additional acetoacetic acid carrier was added to the filtrate. The mixture was refluxed with dilute chromic acid (30) to decompose  $\beta$ -hydroxybutyric acid and carrier acetoacetic acid to acetone and carbon dioxide. Acetone was isolated as Denigès' mercury complex. The mercury complexes were oxidized to carbon dioxide, which was then precipitated as barium carbonate, and  $C^{14}$  was measured. The results are shown in Table V. In general the  $\beta$ -hydroxybutyric acid fraction had approximately twice the activity of acetoacetic acid. 44.4 per cent of the  $C^{14}$  activity originally present in tyrosine was found in ketone bodies after tyrosine had been incubated with liver slices for 2 hours. Relatively little activity was present in the respiratory carbon dioxide. In calculating the percentage conversion of phenylalanine to ketone bodies it has been assumed that only 2 of the

6 ring carbons were used in ketone body formation and the remaining 4 carbons in the benzene ring were used in the formation of radioactive respiratory carbon dioxide. Thus in the metabolism of  $\beta$ -labeled tyrosine little activity would be expected in the respiratory carbon dioxide. This was the case and indicated that little respiratory carbon dioxide came from ketone bodies. The results indicate that the formation of ketone bodies from phenylalanine and tyrosine in rat liver slices represents a major metabolic pathway of these amino acids, and, as expected, the ketone body formation is similar from each amino acid.

*Opening of Benzene Ring*—It is likely that the benzene ring is first broken at only one point and that an 8-carbon dicarboxylic acid is formed. Such a mechanism would be similar to the oxidation of phenol to  $\alpha$ -ketoadipic

TABLE V

*Conversion of Phenylalanine and Tyrosine to Ketone Bodies and Carbon Dioxide*

2.0 mg. of  $C^{14}$  ring-labeled L-phenylalanine ( $3.30 \times 10^6$  counts per minute per mm) and 2.0 mg. of  $C^{14}$ - $\beta$ -labeled L-tyrosine ( $4.26 \times 10^6$  counts) were incubated with approximately 10 gm. of rat liver slices for 2 hours at pH 7.4 and  $38^\circ$ . Inactive acetoacetic acid carrier was added to the system at the end of the reaction.

Initial substrate	Substance isolated at end of reaction	Per cent of original activity found in final product*
Phenylalanine	Respiratory $CO_2$	24.4
	Ketone bodies	56.0
Tyrosine	Respiratory $CO_2$	2.2
	Ketone bodies	44.4

\* See the text for the method used to calculate these values.

acid by a strain of *Vibrio* (37). Further speculation on the details of the mechanism by which two 4-carbon units are split from homogentisic acid is not justified at the present time.

*Metabolism of D-Phenylalanine*—When D-phenylalanine was used in the above experiments, it was found that the activity in the respiratory carbon dioxide and in the ketone bodies was approximately half that found for an equivalent quantity of L-phenylalanine.

*Alanine from Tyrosine (?)*—Recently Felix and Zorn (38) claimed, on the basis of their experiments, that alanine is formed directly from tyrosine in quantitative yields when tyrosine is metabolized by a liver mash. If this view were correct, one would expect  $C^{14}$ - $\beta$ -labeled tyrosine to be converted to  $C^{14}$ - $\beta$ -labeled alanine. To test this theory, 1.3 mg. of the mixed D and L radioactive tyrosine preparation described earlier in this paper were incubated with 5 gm. of rat liver slices. Small aliquots of solution were removed from the reaction mixture at 0, 10, 20, 40, 60, 90, and 120

minutes for filter paper chromatography. Tyrosine and alanine can be separated effectively by means of filter paper chromatography with a solvent consisting of 6 parts of 2,6-lutidine, 1 part of benzyl alcohol, and 3 parts of water. No activity could be detected on the filter paper at the spot where alanine should appear. On the other hand, activity over the tyrosine spot gradually decreased throughout the experiment, and at the end of 2 hours two-thirds of the original activity had disappeared. From this one can conclude that alanine does not accumulate in significant amounts from tyrosine, as suggested by Felix and Zorn.

#### SUMMARY

The work presented in this paper shows that a major pathway in the metabolism of L-phenylalanine and L-tyrosine by rat liver slices results in the formation of two intact 4-carbon units. One unit is a ketone body, and the other malic acid (or its precursor). 2 carbon atoms of the benzene ring, together with 2 carbon atoms of the aliphatic side chain of these amino acids, are converted to ketone bodies. The remaining 4 carbon atoms of the benzene ring form malic acid.

A single experiment with D-phenylalanine showed that this amino acid was metabolized at approximately one-half the rate of the natural isomer.

The belief of Felix and Zorn that tyrosine is metabolized quantitatively to alanine could not be confirmed with tracer studies.

The synthesis of benzaldehyde labeled with  $C^{14}$  in the ring and of phenylalanine labeled with  $C^{14}$  in the ring and  $C^{13}$  in the  $\alpha$  position is reported.

I should like to express my thanks to Dr. H. G. Wood for his contributions of time and advice to this study. I wish to express my appreciation to Mr. J. D. Bordeaux for his aid in the synthesis of labeled phenylalanine. To Dr. J. Meyer I should like to acknowledge my gratefulness for the details of the method for extraction and separation of organic acids from tissues.

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# PRECIPITATION BY TRICHLOROACETIC ACID AS A SIMPLIFICATION IN THE DETERMINATION OF ZINC IN BLOOD AND ITS COMPONENTS

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Recent studies have established the importance of zinc as a biological constituent in human blood. Normal zinc levels for human whole blood, plasma, erythrocytes, and leucocytes have been established (1); significant deviations from the norm occur in leukemia and pernicious anemia, and, possibly, in sickle-cell anemia (2-4). Zinc concentration is considered an expression of the carbonic anhydrase content of erythrocytes (5).

The method employed for zinc analysis in these earlier studies was accurate for samples of biological material containing between 2 and 30  $\gamma$  of zinc, with a standard error of about  $\pm 10$  per cent (6). Its usefulness for the study of blood is limited by the costly and specialized equipment necessary and by the small number of samples which can be analyzed daily.

These objections to the earlier method have now been obviated by protein precipitation with trichloroacetic acid, making zinc quantitatively available for measurement with diphenylthiocarbazone. Zinc determinations, after ashing at 500° (6), have also been made on all samples so studied.

## Procedure

Glassware was cleaned as previously described (6). Water from a Barnstead still<sup>1</sup> was used throughout; repeated tests showed it to be zinc-free.

### Reagents—

1. Diphenylthiocarbazone (Eastman Kodak). A solution of 20 mg. in 200 ml. of  $\text{CCl}_4$ , c.p., stored at 4-6° in the dark; dilutions are made as required.

2. Buffer solution. 1100 gm. of  $\text{Na}_2\text{S}_2\text{O}_8$ , c.p., 180 gm. of  $\text{CH}_3\text{COONa}$ ,

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<sup>1</sup> Manufacturer's catalogue No. SMH-5, Barnstead Still and Steriliser Company, Boston.



C.P., and 20 gm. of KCN, C.P., are dissolved in about 2000 ml. of zinc-free  $H_2O$ . The pH is adjusted to 5.5 by the addition of glacial acetic acid, with use of a Beckman pH meter for control, and the volume brought to 4000 ml. The buffer is extracted with dithizone in a large Squibb separatory funnel, and all the zinc present in the reagents is thus removed.

3. Tartrate solution. 20 per cent  $KNaC_4H_4O_6 \cdot 4H_2O$ , C.P., in distilled water; zinc is extracted as above.

4. Concentrated  $NH_4OH$ .  $NH_4OH$ , C.P., is distilled from an all Pyrex still. Since ammonia extracts zinc, forming a tetra-coordinated complex ion,  $Zn(NH_3)_4^{++}$ , even from Pyrex glassware, it must be freshly prepared at weekly intervals, at least, and stored at low temperature.

5. Chlorophenol red. 0.1 gm. per cent in zinc-free distilled  $H_2O$ .

6. 6 N HCl. Distilled in an all Pyrex still from 6 N HCl, C.P., and stored in clean Pyrex containers.

7. Trichloroacetic acid. Recrystallized and stored at room temperature in a dark paraffin-sealed bottle. 200 ml. of a 20 per cent solution in distilled  $H_2O$  are made up as needed.

*Preparation of Biological Materials*—Human plasma, erythrocytes, and leucocytes were stored in clean Pyrex containers at 4–6°. Aliquots were prepared for zinc analysis by two methods: (1) trichloroacetic acid precipitation of proteins with subsequent analysis of the supernatant fluid, and (2) ashing in platinum crucibles at 500° as described in the earlier method (6).

*Plasma*—Plasma was used undiluted. Lot A was human plasma obtained by centrifugation of fresh whole blood. Lot B was reconstituted dried human plasma.

*Erythrocytes*—Homogeneous samples are necessary, and since it is difficult to obtain such samples of intact erythrocytes, the following procedures were adopted:

Red cells were separated from fresh citrated whole blood by centrifugation; the citrate had previously been extracted with dithizone for zinc. An arbitrary quantity of erythrocytes was spun down in a 15 ml. graduated Pyrex tube, and the volume of packed cells was noted. The small amount of plasma still present was removed. The cells were then washed and spun down three times with a zinc-free isotonic NaCl solution, lyzed with distilled water, transferred to a 200 ml. volumetric flask, and made up to volume with water. In this fashion, homogeneous samples were obtained, the total erythrocyte content of which was known. Several such erythrocyte solutions were prepared containing from 0.141 ml. to 0.232 ml. of red blood cells per ml. of solution.

Alcohol-chloroform precipitation of hemoglobin (7) was used as an alternative method for obtaining homogeneous erythrocyte lysates. This

hemoglobin-free erythrocyte lysate contains zinc as carbonic anhydrase (8), while most of the erythrocyte protein has been removed. This preparation was analyzed.

*Leucocytes*—250 ml. of human leucocytes were obtained by pouring off the buffy coats of citrated blood after the removal of plasma.<sup>2</sup> This suspension contained large numbers of erythrocytes, which were removed by lysis with water. The resulting leucocyte suspension was allowed to stand for 3 weeks at 4°. On microscopic examination the cells had completely disintegrated. Nevertheless, due to coagulation, particulate matter was still present. This suspension was diluted 1:1 with water and dispersed by stirring, and aliquots were used for analysis. Because of the nature of the solution, these aliquots were not homogeneous.

### Method

2 ml. samples of the stock solution were measured into 15 ml. numbered Pyrex centrifuge tubes, and 1 ml. of zinc-free 6 N HCl was delivered into a correspondingly numbered set of Pyrex test-tubes.

To each centrifuge tube, 2 ml. of 20 per cent trichloroacetic acid (TCA) was added, immediately following which a white precipitate formed. The samples were incubated at 90° in a water bath for 5 minutes, with frequent stirring until the precipitates were finely divided. The tubes were cooled and centrifuged at 3000 R.P.M. for 20 minutes. The clear supernatant fluids were decanted into their correspondingly numbered test-tubes and placed in the water bath at 90°.

This procedure, at first, was repeated on the precipitate from one to four times, 2 ml. of 10 per cent TCA being used in all precipitations after the first. A triple precipitation was finally adopted. 2 ml. of 20 per cent TCA were used in the *first* precipitation, and 2 ml. of 10 per cent TCA were used in the *second* and *third* precipitations.

Following incubation for 1 hour at 90°, the combined brownish supernatants were decanted into 125 ml. Squibb separatory funnels. The test-tubes were washed three times with hot distilled H<sub>2</sub>O, which was added to the contents of the separatory funnels.

2 ml. of tartrate solution were placed in the funnels, and the pH was adjusted to approximately 5.5 by adding sufficient amounts of concentrated zinc-free NH<sub>4</sub>OH, with use of chlorophenol red as an indicator. The appearance of a red-violet color was the end-point. 50 ml. of buffer solution were then added. Both a reagent blank and a measured amount of zinc standard solution were analyzed with the daily run of biological samples.

<sup>2</sup> We are indebted to Dr. Robert Pennell of Sharp and Dohme, Glenolden, Pennsylvania, for the generous supply of human leucocytes.

Extraction of zinc with dithizone in carbon tetrachloride was performed as described (6). Carbon tetrachloride solutions were transferred to volumetric flasks and made up to volume. Further dilutions, when necessary for colorimetry, were made directly in the Coleman cuvettes.

*Colorimetry*—The samples were read on a Coleman universal spectrophotometer, model 14, with a minimum of delay after extraction. Dithizone has two absorption maxima, at 625 and at 440  $m\mu$ . The absorption of zinc dithizonate at 625  $m\mu$  is negligible. Zinc dithizonate has an absorption maximum at 525  $m\mu$  and at this wave-length dithizone has an

TABLE I  
*Determination of Ratio of Optical Density of Dithizone at 625  $m\mu$   
to That at 525  $m\mu$ ; Random Dilutions of Dithizone in  $CCl_4$*

$E_{625}^{obs}$	$E_{525}^{obs}$	
0.740	0.180	4.11
0.288	0.075	3.84
0.372	0.100	3.72
0.960	0.225	4.26
0.368	0.094	3.92
0.555	0.142	3.91
0.870	0.215	4.05
0.980	0.243	4.03
0.760	0.182	4.17
1.150	0.276	4.17
0.770	0.190	4.05
0.513	0.131	3.91
0.328	0.087	3.78
Mean.....		3.98
S.D.....		$\pm 0.16$

appreciable absorption. Table I shows the ratio ( $R$ ) of the optical densities of dithizone solutions at 625  $m\mu$  to those at 525  $m\mu$ . This ratio was observed to vary between 3.5 and 4.0 for different batches of dithizone, and was determined routinely with each set of zinc analyses.

The zinc content of each sample is determined from the following equations. Calculations were carried out as previously described (6) and are here presented again for convenience.

$$Zn = E_{525}^{cor.} \times \frac{D \times K}{\frac{100}{v}} \quad (1)$$

where  $Zn$  = zinc in  $\gamma$  in entire sample,  $E_{525}^{cor.} = E_{525}^{obs.} - (E_{625}^{obs.}/R)$ , where

$E_{525}^{\text{obs.}}$  is the optical density observed at 525  $m\mu$ , and  $E_{625}^{\text{obs.}}$  is the optical density at 625  $m\mu$ .  $D$  = dilution of the original extracted volume of dithizone solution.  $v$  = original extracted volume of dithizone solution.  $K$  = a calibration constant, which was determined from the equation

$$K = \frac{E_{525}^{\text{cor.}}}{Zn} \quad (2)$$

A series of zinc standards was extracted with dithizone and read on the Coleman spectrophotometer. By substituting in equation (2)  $K$  was found to be  $62.6 \pm 1.6$  (Table II).

TABLE II  
Determination of Calibration Constant,  $K$

Zinc	$E_{525}^{\text{obs.}}$	$E_{625}^{\text{obs.}}$	$\frac{E_{625}^{\text{obs.}}}{R}$	$E_{525}^{\text{cor.}}$	$\frac{Zn}{E_{525}^{\text{cor.}}} = K$
$\gamma$					
79.1	1.450	0.890	0.222	1.228	64.4
52.7	0.950	0.565	0.141	0.809	65.1
35.2	0.646	0.377	0.094	0.552	63.8
23.4	0.440	0.251	0.063	0.377	62.2
16.6	0.289	0.166	0.042	0.247	63.3
10.4	0.202	0.112	0.028	0.174	59.9
6.95	0.132	0.074	0.019	0.113	61.5
4.64	0.088	0.051	0.013	0.075	61.9
3.10	0.059	0.032	0.008	0.051	60.8
Mean. ....					62.6
S.D. ....					$\pm 1.6$

### Results

**Standards**—Thirty-seven consecutive samples of  $ZnCl_2$  standards, gravimetrically determined as 4  $\gamma$  of zinc, were analyzed by the dithizone method. The results ranged from 3.6 to 4.8  $\gamma$  of zinc with a mean of 4.2  $\gamma$  of zinc and with a standard deviation of  $\pm 0.31 \gamma$ .

**Plasma**—Analysis of a stock of fresh human plasma (Table III) by triple TCA precipitation gave a zinc content of  $2.4 \pm 0.45 \gamma$  per ml. Analysis of the same stock by dry ashing at  $500^\circ$  gave  $1.6 \pm 0.42 \gamma$  of zinc per ml. The stock of reconstituted human plasma, when analyzed by triple TCA precipitation, contained  $3.6 \pm 0.65 \gamma$  of zinc per ml. while by dry ashing it contained  $3.9 \pm 0.99 \gamma$  of zinc per ml. (Table III). 2 ml. samples of plasma were used in the TCA precipitation in both these series. When larger plasma samples (5 ml.) were analyzed by the same procedure,

**TABLE III**  
*Zinc Analysis of Human Plasma by Triple TCA Precipitation and Ashing*

TCA precipitation				Dry ashing		
No. of precipitations	Sample	Total Zn	Zn per ml.	Sample	Total Zn	Zn per ml.
Fresh plasma; Lot A						
	<i>ml</i>	$\gamma$	$\gamma$	<i>ml.</i>	$\gamma$	$\gamma$
3	2	4.6	2.3	2	3.9	1.9
3	2	4.6	2.3	2	4.0	2.0
3	2	3.8	1.9	2	1.7	0.9
3	2	3.1	1.6	2	3.5	1.7
3	2	3.6	1.8	2	3.4	1.7
3	2	6.1	3.0	2	3.6	1.8
3	2	4.1	2.1	2	2.0	1.0
3	2	5.4	2.7	2	2.3	1.2
3	2	4.4	2.2	2	3.4	1.7
3	2	5.6	2.8	2	2.4	1.2
3	2	4.5	2.2	2	4.0	2.0
3	2	5.7	2.9	2	4.8	2.4
3	2	5.7	2.8	2	3.5	1.7
3	2	6.0	3.0	2	3.0	1.5
				2	1.5	0.8
				5	9.1	1.8
				5	9.6	1.9
Mean			2.4			1.6
S.D.			$\pm 0.45$			$\pm 0.42$
Reconstituted plasma; Lot B						
3	2	7.3	3.7	1	4.9	4.9
3	2	7.5	3.7	2	8.2	4.1
3	2	6.5	3.3	2	8.7	4.4
3	2	6.3	3.1	2	8.8	4.4
3	2	5.0	2.5	2	11.3	5.6
3	2	5.2	2.6	5	20.1	4.0
3	2	7.0	3.5	5	19.9	4.0
3	2	6.5	3.3	5	23.5	4.7
3	2	7.7	3.8	5	16.0	3.2
3	2	8.8	4.4	5	19.1	3.8
3	2	6.0	3.0	5	17.7	3.7
3	2	7.9	4.0	5	21.6	4.3
3	2	8.1	4.0	5	18.5	3.7
3	2	9.5	4.8	10	19.0	1.9
3	2	9.1	4.5	10	16.5	1.7
3	2	6.0	3.0			
3	2	7.7	3.9			
Mean...			3.6			3.9
S.D. ....			$\pm 0.65$			$\pm 0.99$

which employs a total of 4 ml. of 20 per cent TCA, the zinc was incompletely recovered. The residual zinc was recovered from the protein residues by ashing. The ashed protein residues of 2 ml. samples contained no zinc.

When amounts of zinc from 2 to 20  $\gamma$  were added as  $\text{ZnCl}_2$  to 2 ml. fresh plasma samples, complete zinc recovery was achieved by routine triple TCA precipitation.

The effect of variation in the number of TCA precipitations is shown in Table IV. 2 ml. samples of fresh plasma were used. Single TCA pre-

TABLE IV  
*Zinc Analysis of Fresh Human Plasma by Single, Double, and  
Quadruple TCA Precipitation on 2 Ml. Samples*

Single precipitation		Double precipitation		Quadruple precipitation	
Total Zn	Zn per ml.	Total Zn	Zn per ml.	Total Zn	Zn per ml.
$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
3.2	1.6	5.7	2.8	6.3	3.1
3.5	1.8	6.6	3.3	4.9	2.5
6.0	3.0	4.5	2.3	4.3	2.1
4.9	2.4	5.0	2.5	5.0	2.5
3.3	1.7	3.7	1.8	4.8	2.4
3.1	1.5	5.0	2.5	5.4	2.6
3.2	1.6	2.9	1.5		
3.4	1.7	2.8	1.4		
		3.1	1.5		
		3.4	1.7		
		4.1	2.1		
		3.7	1.8		
Mean . . . . .	1.9		2.1		2.5
S. D. . . . .	$\pm 0.49$		$\pm 0.56$		
% deviation from triple precipi- tation mean (Table III) . . .	-25		-15		+5

cipitation (2 ml. of 20 per cent TCA) resulted in 75 per cent zinc recovery as compared to triple precipitation, and double TCA precipitation (3 ml. of 20 per cent TCA) gave 85 per cent zinc recovery. Quadruple TCA precipitation gave zinc values 5 per cent above the triple precipitation values. When the protein residues from a single TCA precipitation were merely washed twice with 2 ml. of water and the supernatants combined as usual, no additional zinc was recovered.

*Erythrocytes*—An erythrocyte solution containing 0.141 ml. of packed red blood cells per ml. of solution, when analyzed by triple TCA precipitation, contained  $1.7 \pm 0.22$  gm. of zinc per ml. of solution, corresponding

TABLE V

*Zinc Analysis of Erythrocyte Lysate Containing 0.141 Ml. of Packed Red Blood Cells per Ml. of Sample, Determined by TCA Precipitation and by Ashing*

TCA precipitation				Dry ashing		
Sample	No. of precipitations	Total Zn	Zn per ml.	Sample	Total Zn	Zn per ml.
<i>ml.</i>		$\gamma$	$\gamma$	<i>ml.</i>	$\gamma$	$\gamma$
2	3	3.6	1.8	2	2.6	1.3
2	3	3.4	1.7	2	2.3	1.2
2	3	4.0	2.0	2	1.8	0.9
2	3	3.6	1.8	2	2.3	1.2
2	3	2.4	1.2	2	2.2	1.1
2	3	3.4	1.7	2	2.2	1.1
2	3	3.6	1.8	2	2.1	1.1
2	3	3.5	1.8	2	3.2	1.6
2	3	3.9	1.9	2	3.7	1.8
2	3	3.2	1.6	2	3.5	1.8
2	3	2.8	1.4	2	2.8	1.4
Mean.....			1.7	2	3.1	1.5
S.D.....			$\pm 0.22$	2	3.5	1.8
1	3	2.6	2.6	2	2.8	1.4
1	3	1.7	1.7	2	4.7	2.4
1	3	1.5	1.5	5	8.6	1.7
1	3	2.3	2.3	5	6.2	1.2
1	3	1.7	1.7	1	3.4	3.4
Mean.....			2.0	1	3.9	3.9
2	4	3.8	1.9			
2	4	3.9	1.9			
2	4	2.3	1.2			
2	4	4.6	2.3			
2	4	3.8	1.9			
Mean.....			1.8			
1	4	2.1	2.1			
1	4	1.9	1.9			
Mean...			2.0			1.7
S.D.....						$\pm 0.7$

to 12.1  $\gamma$  of zinc per ml. of packed red blood cells. Ashing of aliquots of this solution gave a value of  $1.7 \pm 0.7$   $\gamma$  of zinc per ml. of solution (Table V).

The effect of varying the size of the sample and the number of TCA precipitations is also shown in Table V.

Table VI shows the results of analysis of a hemoglobin-free erythrocyte lysate by TCA precipitation and by ashing.

TABLE VI  
*Zinc Analysis of Hemoglobin-Free Erythrocyte Lysate (2 Ml. Sample)*

Method of analysis	Total Zn	Zn per ml.
	$\gamma$	$\gamma$
TCA, 1 precipitation	9.1	4.5
	9.2	4.6
TCA, 2 precipitations	9.1	4.6
	10.5	5.2
	10.3	5.2
	9.9	4.9
TCA, 3 precipitations	10.4	5.2
	9.8	4.9
Dry ashing	10.3	5.2
	10.5	5.2

TABLE VII  
*Zinc Analysis of Leucocyte Suspension by Triple TCA Precipitation and by Ashing*

Sample	TCA precipitation		Dry ashing	
	Total Zn	Zn per ml.	Total Zn	Zn per ml.
<i>ml.</i>	$\gamma$	$\gamma$	$\gamma$	$\gamma$
2	6.9	3.5	6.5	3.3
2	6.5	3.3	7.3	3.6
2	8.2	4.1	6.6	3.3
2	10.3	5.1	15.5	7.7
2	6.2	3.1	9.6	4.8
2	6.5	3.2	14.7	7.3
2	7.9	4.0	4.3	2.2
2	5.4	2.7	11.3	5.6
10			25.2	2.5
10			24.7	2.5
Mean . . . . .		3.6		4.3
S.D. . . . .		$\pm 0.7$		$\pm 1.8$

*Leucocytes*—Triple TCA precipitation gave  $3.6 \pm 0.7 \gamma$  of zinc per ml. of suspension; ashing gave  $4.3 \pm 1.8 \gamma$  of zinc per ml. (Table VII).

#### DISCUSSION

Before any data obtained with the TCA precipitation could be evaluated, contamination of reagents and glassware had to be controlled by measure-



ments of "blanks." The blank was composed of all chemicals in the proportions and quantities used in the analysis of the biological samples, with randomly selected glassware. A "standard" was designed to check the reproducibility of experimental conditions from day to day, and the extraction of a 4  $\gamma$  standard was taken as evidence that no variation in the standard solution, the extraction procedure, or the colorimetry had occurred. The standard in thirty-seven consecutive samples was  $4.2 \pm 0.31 \gamma$ .

When the supernatant from the TCA-precipitated proteins is shaken with dithizone in  $\text{CCl}_4$ , the  $\text{CCl}_4$  phase is emulsified in the  $\text{H}_2\text{O}$  phase. It collects only incompletely into a separate phase after a period of several hours. Under these conditions quantitative extraction of zinc is not possible. Incubation of the supernatant with 1 ml. of 6 N HCl prior to extraction results in complete separation of the two phases. Presumably, inadequate phase separation is due to protein degradation products of high molecular weight, which are further reduced in size by HCl.

We have substituted chlorophenol red for methyl red, which was used previously in this technique (6). Methyl red is extracted into the  $\text{CCl}_4$  phase and its colorimetric absorption may cause positive errors in zinc determination. Moreover, the methyl red range lies between pII 4.4 (red) and 6.0 (yellow), and the "peach color" supposed to occur at pH 5.5 is not distinct and is subject to observational error. On the contrary, chlorophenol red is not  $\text{CCl}_4$ -soluble and abruptly changes from yellow to violet at pH 5.5, the critical pH of the procedure.

The results of the analyses of blood components by triple TCA precipitation show excellent recovery of zinc. The standard deviations for the TCA series and for ashing controls are comparable. It is apparent that the standard deviations of the ashing series are greater, in all instances but one, than those obtained by TCA precipitation. The consistently narrower spread of the TCA values indicates better reproducibility. Still better reproducibility could be obtained in both the TCA and the ashing methods when larger samples were analyzed, but for ease of handling and for economy of materials small samples seemed preferable.

The mean values obtained by these two methods are not significantly different statistically, except in the case of fresh human plasma, for which the mean value by triple TCA precipitation is significantly higher than the ashing mean. We are unable to explain the discrepancy in this one series. Contamination was excluded as a source of error, but it is possible that zinc was lost from the ashed samples by volatilization.

That all the zinc was recovered by TCA precipitation is further demonstrated by the complete recovery of known amounts of zinc added to plasma samples, and by the absence of zinc from the protein residues after triple acid precipitation. It is realized that these series presented are small for

statistical evaluation. However, the means and standard deviations for plasma and erythrocytes are in good agreement with the larger series previously obtained by ashing (1).

With single precipitation only 75 per cent, and with double precipitation 85 per cent, of the total zinc in 2 ml. samples was recovered. Quadruple precipitation, however, resulted in no appreciably greater zinc recoveries than triple precipitation. When 5 ml. samples were triply precipitated with the same amounts of TCA as used on 2 ml. samples, not all the zinc was recovered; the deficit could be demonstrated, by ashing, to be in the protein residue. It is, therefore, evident that a total of 4 ml. of 20 per cent TCA is not adequate to split off all the zinc from 5 ml. samples of the type of biological substrate employed in these studies. For 2 ml. samples, triple precipitation is optimal.

In the case of the hemoglobin-free erythrocyte lysate, 2 ml. samples treated by *double* acid precipitation showed zinc values identical with similar triply precipitated samples, or ashed samples. These samples had a smaller amount of protein residue than the other materials handled.

The data presented point to a possible relationship between the amount of TCA necessary for complete zinc liberation and the protein content of the sample. Though these data were not specifically designed to answer this question, it would seem that this is indicated, on one hand, by the smaller amount of TCA necessary to liberate zinc from samples of low protein content (hemoglobin-free erythrocyte lysate), and, on the other hand, by the incomplete recovery of zinc from larger samples.

The greatest variation in results is noted in both the TCA-precipitated and the ashed leucocyte samples, although the means are comparable. This stock solution was noted to contain undissolved material, and was not homogeneous, giving aliquots that were not strictly comparable. They were, nevertheless, utilized to demonstrate complete zinc recovery from white blood cells by the TCA method.

It has been shown that zinc exists in blood components in several states (3), and it is noteworthy that this TCA procedure will liberate zinc quantitatively from its various forms of combination in plasma, erythrocytes, and leucocytes. This may be of value in elucidating the manner of binding of zinc in zinc proteins.

As has been pointed out, the ashing technique requires expensive platinum crucibles and muffle furnaces. The time-consuming nature of the procedure, as well as the expense, limits its general applicability to clinical and research work. The TCA method requires no specialized equipment. It is more reproducible, and therefore more accurate, than the ashing procedure. Double the daily number of analyses, or more, can be performed without additional effort.

## SUMMARY

1. A method for zinc analysis by TCA precipitation applicable to erythrocytes, leucocytes, and plasma is presented.

2. The technique is accurate for samples containing as little as 1  $\gamma$  of zinc.

3. The procedure is more accurate than dry ashing and is more rapid; it is well suited for general clinical use.

We wish to express our gratitude to Dr. John R. Loofbourow for the advice and encouragement he has given so unstintingly.

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# THE UTILIZATION OF THE NITROGEN OF AMMONIUM SALTS, UREA, AND CERTAIN OTHER COMPOUNDS IN THE SYNTHESIS OF NON-ESSENTIAL AMINO ACIDS IN VIVO\*

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Inasmuch as ten of the amino acids present in proteins may be synthesized readily by the organism of the rat (1), it becomes of interest to determine what influence would be exerted upon the growth of the animals by restricting the intake of dietary nitrogen to that present in the essential amino acids when each is furnished at the minimum level compatible with maximum growth (*cf.* (2)). It should be recalled that these minimum levels were established under conditions which provided an abundance of all amino acids other than the one under investigation. It might be anticipated that a diet carrying the essentials only, each at its minimum level, would lead to an inhibition in growth, since, under these circumstances, nitrogen would not be available for the manufacture of the non-essentials. In other words, the latter, under the conditions specified, might be expected to become the limiting factors in the growth of the subjects. In that event, an opportunity would be afforded of determining what types of compounds may serve as sources of nitrogen for synthetic purposes in the body.

Such were the considerations which led to the investigation outlined in the following pages. The results demonstrate that growth is markedly diminished when the ration furnishes only the essential amino acids at their minimum levels, and that a striking acceleration in the rate of gain occurs when nitrogen is supplied in the form of any one of a number of compounds.<sup>1</sup>

\* Aided by grants from the United States Public Health Service and the Graduate College Research Fund of the University of Illinois.

† Part of the experimental data in this paper are taken from a thesis submitted by Leonard C. Smith in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the University of Illinois.

<sup>1</sup> Many of the data herein recorded were presented by the senior author as part of a symposium paper sponsored by the American Institute of Nutrition in Detroit on April 21, 1949. In the meantime, a preliminary report involving somewhat similar experiments with diammonium citrate in a small number of animals has been published by Lardy and Feldott (3).

## EXPERIMENTAL

Except in certain of the nitrogen balance experiments described below, male weanling rats have served as the subjects. Each animal was housed in a separate cage, and was permitted to consume food and water *ad libitum*. All growth tests were continued for 28 days. The amino acids were purified invariably until they yielded correct analytical values.

In Table I is presented the composition of two amino acid mixtures

TABLE I  
*Composition of Amino Acid Mixtures*

	Mixture XXVI		Mixture XXVII	
	Physio- logically active	As used	Physio- logically active	As used
	gm.	gm.	gm.	gm.
Valine . . . . .	0.7	1.40*	0.35	0.35
Leucine . . . . .	0.8	0.80	0.40	0.40
Isoleucine . . . . .	0.5	1.00*	0.25	0.25
Methionine . . . . .	0.6	0.60*	0.30	0.30*
Threonine. . . . .	0.5	1.00*	0.25	0.25
Phenylalanine . . . . .	0.9	0.90*	0.35	0.35*
Tryptophan . . . . .	0.2	0.20	0.10	0.10
Lysine . . . . .	1.0		0.50	
monohydrochloride . . . . .		1.25		0.63
Histidine . . . . .	0.4		0.20	
monohydrochloride monohydrate . . . . .		0.54		0.27
Arginine . . . . .	0.2		0.10	
monohydrochloride . . . . .		0.24		0.12
Sodium bicarbonate. . . . .		0.80		0.44
	5.8	8.82	2.80	3.46

\* Racemic acids.

which were employed in the experiments. Both contained the ten essentials only. Mixture XXVI, when included in the food to the extent of 8.82 per cent, furnished each amino acid at the minimum level. As will be observed, five of the amino acids were racemic products. Three of these, namely valine, isoleucine, and threonine, were doubled in quantity in the mixture as used in order to provide the requisite amount of each L-amino acid. However, this was not necessary in the case of methionine and phenylalanine, since their D and L isomers are equally effective for growth in the rat (4, 5).

The ratio of components in Mixture XXVI is slightly modified in three particulars from similar values presented elsewhere (2) about 12 years ago.

Subsequent tests on many additional animals indicated that the minimum intakes of leucine and threonine for maximum growth, under the conditions of our experiments, were nearer 0.8 and 0.5 gm. respectively per 100 gm. of food than the values of 0.9 and 0.6 gm. originally suggested. Furthermore, detailed evidence has already been presented (5) to the effect that the minimum phenylalanine requirement is 0.9 gm. rather than 0.7 gm. per 100 gm. of ration. Thus, the sum of the physiologically active amino acids remains at 5.8 gm., although three individual values have been altered in the light of more extensive experience.

Mixture XXVII contained half the minimum level of each amino acid other than phenylalanine. The newer figure for the latter had not been established when the experiments involving this mixture were conducted. Consequently, half of the original value of 0.7 gm. was employed. This is unimportant inasmuch as the phenylalanine content was sufficient for the purposes for which the mixture was used. In addition to the lower levels of the amino acids, Mixture XXVII differed from Mixture XXVI in that it contained only physiologically active components. To accomplish this, valine was resolved according to the method of Fischer (6). All of the threonine and most of the isoleucine were isolated from proteins. Part of the isoleucine was prepared by resolution of the racemic form by the procedure of Locquin (7).

In Table II is shown the composition of Diets 1 and 2 containing Mixtures XXVI and XXVII respectively. Both diets were appropriately supplemented with vitamins. Each kilo of Diet 1 was thoroughly admixed with the vitamins listed in the second column of Table III. With Diet 2, the vitamins and concentrates enumerated in the third column of Table III were administered apart from the basal ration in the form of two pills daily, each containing half of the indicated daily intake. The unknown nitrogen present in the liver extract did not exceed 3.5 mg. daily with Diet 1 and 2.0 mg. daily with Diet 2.

Attention was first directed to the growth of animals upon Diet 1 and the effects of adding several compounds as supplements to this diet. With each supplement except sodium citrate, an amount was used which furnished 0.574 gm. of nitrogen, as determined by analysis. This figure was selected arbitrarily because it represents the difference between the nitrogen content of Mixture XXVI and that of the physiologically active amino acids of Mixture XXIII. As explained elsewhere (1), Mixture XXIII contains all of the amino acids known to be present in proteins except citrulline, and has been used in hundreds of tests in this laboratory. It is now regarded as our standard.

Numerous preliminary experiments demonstrated that animals are able to grow slowly when they receive Diet 1 alone. In order to obtain

TABLE II  
*Composition of Basal Diets*

	Diet 1	Diet 2
	gm.	gm.
Amino acid Mixture XXVI	8.82	
“ “ “ XXVII		3.46
Sucrose ..	15.00	15.00
Dextrin ..	67.43	64.54
Cellu flour.	2.00	
Agar.....		2.00
Salt mixture..	4.00*	3.00†
Corn oil..	2.00	4.00
Haliver oil..	0.05	
Cod liver oil...		4.00
Inositol. ....	0.10	
Choline chloride	0.20	
Lard.....		4.00
Liver extract‡.	0.40	
	100.00	100.00

\* Jones and Foster (8).

† Hubbell, Mendel, and Wakeman (9).

‡ Wilson's liver powder, 1:20.

TABLE III  
*Vitamin Supplements*

	Added to each kilo of Diet 1	Daily intakes of animals upon Diet 2*
	mg.	γ
Thiamine hydrochloride.....	5	20
Riboflavin.....	10	40
Pyridoxine hydrochloride ..	5	20
Nicotinic acid. ....	5	20
Calcium pantothenate.....	25 (d)	200 (dl)
		mg
p-Aminobenzoic acid ..	300	3
α-Tocopherol.....	25	
Wheat germ oil ..		50
2-Methyl-1,4-naphthoquinone	2	
	γ	
Biotin. ....	100	
Inositol.....		10
Choline chloride..		10
Wilson's liver powder, 1:20. ....		25

\* Upon this diet the vitamin supplements were administered in the form of two pills daily, each containing half of the quantities listed.

a more accurate indication of the magnitude of the gain and of the improvement, if any, in the nutritive quality of the food which results from the inclusion of an additional source of nitrogen, the experiments summarized in Table IV were carried out. In Series I, a comparison was made of growth upon Diet 1 without and with the addition of diammonium citrate. For this purpose, thirty-two male rats from ten litters were divided as equitably as possible between the two groups. As will be observed, the sixteen animals which received Diet 1 alone showed a mean gain of  $42.1 \pm 0.86$  gm., while their litter mates which received the same basal ration supple-

TABLE IV

*Growth on Minimum Levels of Essential Amino Acids without and with Nitrogenous Supplements*

The experiments covered 28 days each.

Series No.	No. of animals (males)	Mean gain in weight and probable error of mean	Nature of diet*
		gm.	
I	16	$42.1 \pm 0.86$	Diet 1
	16	$80.6 \pm 1.59$	" 1 + diammonium citrate
II	15	$91.0 \pm 1.65$	" 1 + " "
	15	$63.6 \pm 1.89$	" 1 + urea
III	15	$85.3 \pm 1.27$	" 1 + diammonium citrate
	15	$70.9 \pm 1.11$	" 1 + glycine
IV	15	$93.9 \pm 2.30$	" 1 + diammonium citrate
	14	$91.6 \pm 1.42$	" 1 + L-glutamic acid
V	10	$83.4 \pm 1.67$	" 1 + diammonium citrate
	10	$39.4 \pm 1.27$	" 1 + trisodium citrate
VI	10	$83.6 \pm 0.97$	" 1 + ammonium acetate

\* Each nitrogenous supplement furnished 0.574 gm. of nitrogen per 100 gm. of food. In Series V, the ammonium citrate and sodium citrate were supplied in equivalent quantities.

mented with diammonium citrate manifested a mean gain of  $80.6 \pm 1.59$  gm.

Several factors may have contributed to the ability of the animals to gain upon the unsupplemented ration. As pointed out above, the ration contained three racemic amino acids. One cannot exclude the possibility that the D forms of valine, isoleucine, and threonine, though unavailable as such for tissue synthesis, may have served as sources of nitrogen for the manufacture of the non-essentials. This explanation seems unlikely, inasmuch as such a use of the amino acids would have involved their deamination. Unpublished experiments in this laboratory have shown that D-valine and D-isoleucine are incapable of replacing the L



isomers for growth purposes in the rat (*cf.* (2)). On the other hand, the corresponding  $\alpha$ -keto analogues of these amino acids promote excellent growth,<sup>2</sup> probably by undergoing asymmetric amination. Thus, the inability of the organism to invert D-valine and D-isoleucine may be attributable to its incapacity to accomplish their deamination.

A more reasonable explanation of the moderate growth of animals on Diet 1 is the reutilization of ammonia liberated in tissue catabolism (*cf.* Schoenheimer, Ratner, and Rittenberg (10)). Furthermore, as has been pointed out above, the amino acids in Mixture XXVI reflect the requirements of the rat for *maximum* growth when the diet furnishes the non-essentials. In the absence of the latter, the metabolism of the essentials may represent a compromise between direct utilization and decomposition with the liberation of ammonia for the manufacture of the non-essentials. Thereby, all amino acids might become available, but not in adequate amounts to meet the requirements for rapid growth. In any event, the data in Series I demonstrate clearly that, under the conditions in question, the supply of non-essential amino acids, or more specifically of nitrogen for their synthesis, becomes the limiting factor. This concept is confirmed by the observation that the inclusion in the food of diammonium citrate induces a marked increment (38.5 gm.) in the mean gain of the subjects. This substantiates, by an entirely different technique, the finding of Foster, Schoenheimer, and Rittenberg (11) that dietary ammonia can be utilized for amino acid synthesis. Moreover, our data reveal the further fact *that the reactions involved in these synthetic processes take place with remarkable facility*, as indicated by the pronounced acceleration in the rate with which the subjects gain in body weight.

Having demonstrated the growth stimulation of diammonium citrate, like experiments were carried out with other nitrogenous supplements. Series II, III, and IV (Table IV) illustrate the effects of urea, glycine, and L-glutamic acid, respectively. Diammonium citrate was used for comparison throughout, and the animals of the two groups within each series were always litter mates. As will be observed, L-glutamic acid is just as effective a source of nitrogen as is diammonium citrate. On the other hand, glycine is less satisfactory, and urea is the least active of the three. However, even urea can serve as a fairly potent donor of nitrogen for the synthesis of the non-essentials. A comparison of the mean gain of the rats which consumed urea (Series II) with those which received the basal diet alone (Series I) shows a difference of 21.5 gm. This is statistically highly significant. Moreover, many other experiments not included in the present paper demonstrate that a mean gain of

<sup>2</sup> Rose, W. C., Johnson, J. E., and Hancock, E. M., unpublished data.

40 to 42 gm. in 28 days is typical of weanling rats of our colony receiving Diet 1 without nitrogenous supplementation.

In view of the fact that the two most readily utilized supplements in the above experiments, namely diammonium citrate and L-glutamic acid, are closely related to intermediates in the Krebs tricarboxylic acid cycle, it appeared necessary to exclude the possibility that the growth effects might be due in part to the non-nitrogenous portions of these molecules. For this purpose, a comparison was made of the effects of trisodium citrate and diammonium citrate. The concentration of citrate ions was identical in the two diets. The results are summarized in Series V, Table IV. Evidently, the citrate ion is not the active agent, since the mean gain of the subjects which received sodium citrate is comparable to that of animals upon the basal diet alone.

Finally, ten animals (Series VI) received Diet 1 supplemented with ammonium acetate. Because of the instability and hygroscopic nature of this salt, a concentrated solution was prepared, analyzed for nitrogen, and added to the basal ration in an amount sufficient to furnish the required quantity of nitrogen. No control experiments were conducted, but the results (Table IV) indicate that this salt is quite satisfactory as a source of nitrogen, despite its ammoniacal odor. Indeed, the mean gain of the subjects was within the range of that observed in other series when diammonium citrate was used as the supplement.

In order to obtain further evidence regarding the utilization of urea and diammonium citrate, several nitrogen balance experiments were carried out upon rats. Tests were first conducted to establish what intake of a mixture of the ten essentials would just suffice for body weight maintenance. The results showed that 50 per cent of the minimum levels then in use (the minimum for phenylalanine at that time being regarded as 0.7 gm. per 100 gm. of food, as explained above) would permit approximate body weight equilibrium. Therefore, this ratio of the component amino acids was employed in formulating Mixture XXVII (Table I). This mixture was incorporated in an otherwise adequate diet (Diet 2, Table II), except for certain vitamins which were administered separately. The urea and diammonium citrate were added in amounts sufficient to double the nitrogen content of the food.

The animals were housed in cages with  $\frac{3}{4}$  in. wire screen bottoms. These were supported by metal funnels, the inner surfaces of which were coated with paraffin. To collect the feces, a fine mesh screen was placed a few inches above the inverted apex of each funnel. The feces were removed each day, acidified slightly with sulfuric acid, and preserved under toluene. At the same time, the funnels were washed down with a stream of 2 per

cent boric acid solution. The combined urine samples and washings from each animal were strained through a small cotton plug, preserved under toluene, and stored at low temperature until analyzed. Nitrogen was determined in the food and, at 4 day intervals, in the urine and feces by the Scales and Harrison (12) modification of the Kjeldahl procedure.

In Table V are summarized the results of three such experiments, each extending over a period of 96 days. The three animals, one male and two females, had received the basal ration (Diet 2) for 28 days prior to the collection of the excreta. During that time, they showed weight

TABLE V

*Nitrogen Balance without and with Urea or Diammonium Citrate in Diet*

Rat No. and sex	Period	Average daily food intake	Average daily N			Weight change for period	Nature of diet*
			In-take	Out-put	Balance		
	days	gm.	mg.	mg.	mg.	gm.	
5188 ♀	28	4.0	20.1	17.2	+2.9	+1	Diet 2
	28	5.4	47.6	28.8	+18.8	+24	" 2 + 0.85% urea
	12	4.3	20.4	20.3	+0.1	-5	" 2
	28	6.9	59.7	29.3	+30.4	+40	" 2 + 3.2% diammonium citrate
5189 ♀	28	4.0	20.0	18.4	+1.6	-2	" 2
	28	4.8	42.4	26.1	+16.3	+22	" 2 + 0.85% urea
	12	4.7	22.1	19.9	+2.2	-2	" 2
	28	6.1	53.4	29.8	+23.6	+33	" 2 + 3.2% diammonium citrate
5190 ♂	28	3.6	18.2	17.3	+0.9	0	" 2
	28	4.8	42.6	26.8	+15.8	+17	" 2 + 0.85% urea
	12	3.8	18.7	19.7	-1.0	-4	" 2
	28	5.6	49.3	26.0	+23.3	+26	" 2 + 3.2% diammonium citrate

\* The urea or diammonium citrate was added in an amount sufficient to double the nitrogen content of the food.

changes of +1, -1, and +1 gm., respectively. Examination of the data in Table V reveals the pronounced effect of both urea and diammonium citrate upon nitrogen balance and body weight. Without either supplement, the nitrogen balance varied from +2.9 to -1.0 mg. per day, and the body weight from +1 to -5 gm. per period. The addition of either source of extra nitrogen induced a strong retention of this element, which was accompanied by a slow growth of the subjects. As in the experiments with Diet 1, so here also the ammonium salt exerted a more pronounced effect than did urea. However, both compounds must have been used as sources of nitrogen for the synthesis of amino acids. One may affirm with confidence, therefore, that in the rat the nitrogen of the non-essential amino acids may be derived from a variety of com-

pounds, including ammonium salts, certain amino acids, and even urea. The effectiveness of these supplements in promoting growth and nitrogen retention probably is dependent merely upon the facility with which they can be converted by the cells into ammonia for the amination of appropriate keto acids, such as pyruvic, oxalacetic, or  $\alpha$ -ketoglutaric acid, or their ability to participate, as in the case of L-glutamic acid, in transamination reactions. Such may be the explanation for the somewhat less pronounced effects of urea and glycine, as contrasted with the other compounds tested in this investigation.

In conclusion, the reader should be reminded that the results herein reported are not to be confused with the well known fact that in ruminants urea and ammonium salts serve as rather satisfactory supplements for low protein diets (13-15). In these species, microorganisms in the rumen appear to be responsible for the synthesis of proteins, and hence of the component amino acids. Under like dietary conditions, non-ruminating animals are said to respond little, if at all, to the inclusion of urea in the food (*cf.* Kriss and Marcy (16)). It should be emphasized that the synthetic processes recorded in the present paper involve the non-essentials only, and that the essential amino acids must be furnished preformed. Whether alimentary microorganisms play a rôle in the rat cannot be determined from the data herein described, but will be considered in a subsequent paper.

#### SUMMARY

Weanling rats gain very slowly upon a diet in which virtually all of the nitrogen is in the form of the ten essential amino acids, each at the lowest level compatible with maximum growth when accompanied by abundant supplies of the non-essentials. Under such circumstances, the limiting factor is the inability of the cells to synthesize adequate amounts of the non-essentials because of the shortage of nitrogen.

The addition to such a ration of ammonium salts, L-glutamic acid, glycine, or even urea induces a marked acceleration in the rate of gain. Of these supplements, ammonium salts and L-glutamic acid are the most effective, glycine is intermediate, and urea is the least active. These variations in potency are doubtless to be accounted for by differences in the facility with which the compounds in question may serve as sources of nitrogen for amination or transamination reactions.

Young rats receiving a diet containing only half the minimum levels of the essential amino acids maintain nitrogen equilibrium and body weight. The addition of either urea or diammonium citrate induces strong nitrogen retention accompanied by an increase in body weight.

Both the growth tests and the nitrogen balance studies demonstrate

clearly that the nitrogen of ammonium salts, certain amino acids, and urea can be utilized by the rat under the conditions specified.

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# ENZYMATIC HYDROLYSIS AND OXIDATION OF MONOTHIOPHOSPHATE\*

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As a part of a program of study of the mechanism of oxidation of organic sulfur, various phosphorylated derivatives of sulfur compounds have been prepared. These derivatives were investigated as to their behavior in isolated tissue systems. The present report deals with a study of the behavior of monothiophosphate.

## EXPERIMENTAL

*Preparation of Disodium Thiophosphate*—Disodium thiophosphate was conveniently prepared by adding a solution of sodium hydroxide (6 N) to 50 ml. of thiophosphoryl chloride ( $\text{SPCl}_3$ ) in a flask fitted with a stirrer and cooled in an ice bath. Sodium hydroxide was added, with prolonged stirring, until the mixture was barely alkaline to phenolphthalein. The hydrate was precipitated when alcohol was added to make a final concentration of about 50 per cent. After two recrystallizations from water, the material was free of chloride and orthophosphate and the over-all yield was about 70 per cent. Total phosphate was found to be 8.3 per cent. The theoretical value was 8.3 per cent, calculated as P for  $\text{Na}_2\text{HSPO}_3 \cdot 12\text{H}_2\text{O}$ .

*Acid Hydrolysis of Disodium Thiophosphate*—The thiophosphate did not respond to tests for orthophosphate<sup>1</sup> (1) but, with acid hydrolysis, all the

\* These studies were supported by a grant from the United States Public Health Service.

<sup>1</sup> The author has found that, in his experience, the method of Allen (1) is superior to other methods in the determination of orthophosphate, phosphate of acid-labile esters, and total phosphate. The procedure for orthophosphate and total phosphate is described in the original article (1). The procedure for acid-labile esters of phosphate, more or less identical with that usually employed, is as follows: The samples are diluted to 10 ml. with water, and 2 ml. of 60 per cent perchloric acid are added. The tubes are heated in a boiling water bath (12 minutes at or near sea level; 18 minutes at the altitude of this laboratory, 5000 feet) and are then cooled to room temperature. The estimation of orthophosphate in the cooled tubes is made as described by Allen. With such compounds as adenosine triphosphate, the acid-labile phosphate, as determined by this method, is identical with that determined by a procedure involving hydrolysis with 1 N sulfuric acid, followed by determination of the orthophosphate by the method of Fiske and Subbarow (2). The particular advan-

phosphate was released. In Fig. 1, it is seen that the thiophosphate was somewhat less labile to acid hydrolysis than was pyrophosphate.

*Determination of Thiophosphate*—As illustrated in Fig. 1, the behavior of thiophosphate during hydrolysis with acid was not sufficiently distinct from other "acid-labile" phosphates to provide a method of differentiation. It was found possible, however, to determine the thiophosphate as ortho-

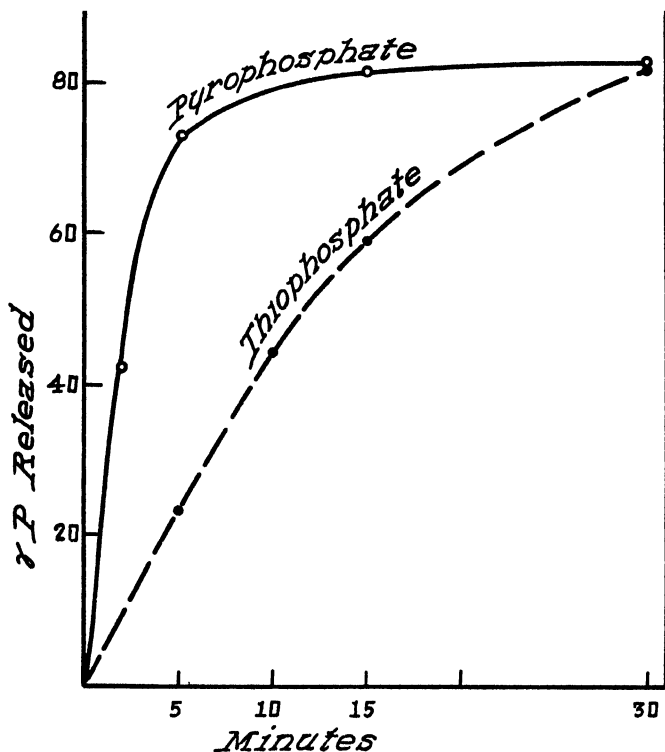


FIG. 1. Samples containing 1 mg. of monothiophosphate (83  $\gamma$  of P) were heated in a boiling water bath (94°) with 1 N perchloric acid. The samples were cooled and orthophosphate was determined by the method of Allen (1).

phosphate following treatment with an aqueous solution of bromine. In Table I, recoveries of thiophosphate are given and it is seen that theoretical recoveries were obtained. The phosphate of adenosine triphosphate was not converted to orthophosphate by the treatment with bromine water; other compounds in which orthophosphate is released by hydrolysis with acid have not been tested.

tages of the method are that calcium and barium ions do not interfere with the determinations and the same reagents may be used for the determination of ortho-, acid-labile, and total phosphate.

*Enzymatic Hydrolysis of Thiophosphate*—Kidney and liver of rats were investigated as possible sources of a hydrolytic enzyme. In Table II, the

TABLE I

*Estimation of Thiophosphate*

To the sample in 5 ml. of solution was added 1 ml. of saturated solution of bromine and, after 10 minutes at room temperature, orthophosphate was determined by the method of Allen (1) without preliminary destruction of excess bromine.

P added as thiophosphate	P recovered as orthophosphate
$\gamma$	$\gamma$
8.4	8.5
16.8	17.0
42.0	42.0
84.0	84.0

TABLE II

*Enzymatic Hydrolysis of Thiophosphate*

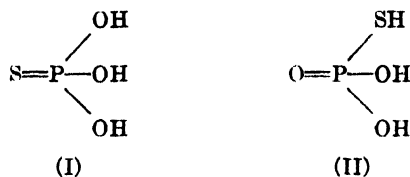
10 mg. of disodium thiophosphate were incubated for 1 hour with 2 ml. of extract of kidney or liver tissue (1 gm. of tissue in 10 ml. of saline) in a total volume of 10 ml. of 0.05 M diethyl barbiturate, pH 8.5. Various activators were added to make a final concentration of 0.001 M. Orthophosphate was determined by the method of Allen (1). In the column, "Non-enzymatic hydrolysis," results obtained in control experiments are recorded. In these control experiments, boiled extract was added; all values are corrected for orthophosphate released by the incubation of the substrate and the enzyme preparations alone.

Tissue	Activator	P released	Non-enzymatic hydrolysis	
			Boiled extract	No extract
Kidney	$\gamma$			
	None	110		
	Mg <sup>++</sup>	170		
	Cu <sup>++</sup>	530	560	1130
	Zn <sup>++</sup>	310	310	820
	Mn <sup>++</sup>	150		
	Co <sup>++</sup>	110		
	Citrate	60		
Liver	Cyanide	60		
	None	90		
	Cu <sup>++</sup>	390	390	
	Zn <sup>++</sup>	240	240	

results of these studies are given; the effects of various ions are also illustrated. In contrast to other ions, zinc and cupric ions bring about the non-enzymatic hydrolysis of thiophosphate. The difference between the hydrolysis in the presence and in the absence of boiled extract may be in-



terpreted as a measure of the combination of the cupric and zinc ions with the denatured protein. It appears possible that this phenomenon may be found to be useful in the development of a method of determination of the combining power of proteins with these ions. The following interpretation of the hydrolysis of thiophosphate by cupric and zinc ions is offered. It may be assumed that there are two isomeric forms of thiophosphate and the isomeric form (II) is capable of forming metal complexes.



In the case of cupric and cobaltous ions, the formation of such complexes is indicated by a many-fold enhancement of the color of the solutions of the ions upon the addition of monothiophosphate. The breakdown of these metal complexes, in the case of zinc and cupric ions, is driven to completion by the formation of the insoluble sulfides.

*Oxidation of Thiophosphate*—A comparative study was made of the oxidation of thiophosphate and sodium sulfide by homogenates of kidney tissue. It was found that sulfide was oxidized by the boiled homogenate and cytochrome *c*; fresh homogenate was without further effect. However, thiophosphate was oxidized by the fresh homogenate and not by the boiled homogenate. These studies are summarized in Fig. 2.

Thiophosphate was found to be oxidized by homogenates of liver tissue at a rate and extent similar to that of kidney tissue. As with kidney tissue, there was a non-enzymatic oxidation of sulfide by boiled homogenates of liver tissue and cytochrome *c*. Maximal uptakes of oxygen (in 5 to 8 hours), in the case of thiophosphate, corresponded to an uptake of 1 mole of oxygen per mole of thiophosphate or to a valence change of 4; such a valence change is compatible with the formation of thiosulfate. When the filtrates were titrated with iodine (addition of standard iodate, potassium iodide, and sulfuric acid, followed by back-titration with thiosulfate), the values were found to correspond to the calculated concentration of thiosulfate. It is to be emphasized that such a determination does not establish the presence of thiosulfate but it is difficult to reconcile the uptake of oxygen and the iodometric titration without the assumption that thiosulfate was produced; tests for sulfate were essentially negative.

*Possible Occurrence of Thiophosphate in Urine*—No thiophosphate was detectable in normal urine but, following the ingestion of cysteine, a possible excretion of thiophosphate was observed. A study of the excretion of

cysteine and thiophosphate following the ingestion of cysteine is summarized in Table III.

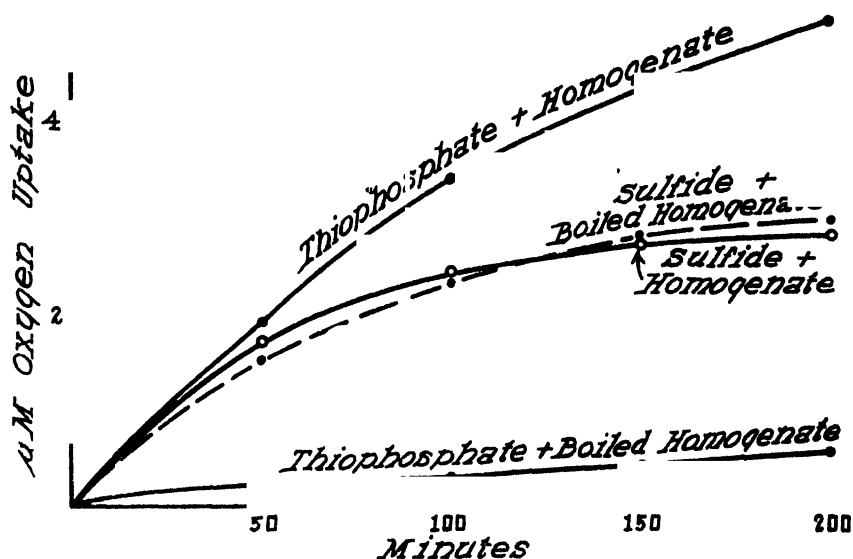


FIG. 2. 10  $\mu\text{M}$  of substrate were present in each flask and the procedure followed was that described by Umbreit *et al.* (3) for the study of oxidative phosphorylation, except that succinate, adenosine triphosphate, and creatine were omitted.

TABLE III

*Possible Excretion of Thiophosphate*

10 gm. of cysteine were ingested by an 80 kilo male, and urine samples were collected at the intervals indicated. Acid-labile and thiophosphate phosphorus were determined as described earlier in the text.

Time after ingestion	Inorganic P	Acid-labile P	Thiophosphate P	Cystine (Sullivan and Hess (4))
<i>hrs.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>
Normal	61	0	0	0.3
2	27	4	4	6.3
3.5	29	2	2	8.8
5	35	3	3	4.5
9	49	2	2	1.9
22	47	1	1	0.7

DISCUSSION

If the possible excretion of thiophosphate following the ingestion of cysteine is neglected, no evidence is offered for the occurrence of thiophos-

phate in normal metabolism but, since thiophosphate is hydrolyzed and oxidized by tissues, it merits further consideration as a possible intermediate in the oxidation of sulfur. Even though sulfide is released from cysteine by the action of an enzyme of the liver, there is evidence that sulfide is poorly utilized by tissues. As noted above, sulfide is oxidized poorly, if at all, by homogenates of kidney or liver and, according to a recent report (5), is not utilized in the synthesis of cysteine by mutants of *Neurospora*.

#### SUMMARY

A method has been developed for the detection of monothiophosphate. The enzymatic hydrolysis and oxidation of thiophosphate by tissues of the rat were studied; thiophosphate was found to be hydrolyzed and oxidized by kidney and liver tissue of the rat. A possible excretion of thiophosphate by man following the ingestion of cysteine was observed.

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# DETERMINATION OF ALCOHOL BY MICRODIFFUSION

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Numerous methods have been described for the determination of alcohol in small amounts of blood, and an extensive bibliography of such methods has been given by McNally and Coleman (1). Most procedures depend upon the volatility of alcohol and its power to reduce oxidizing agents.

The microdiffusion principle, applied in units designed by Conway (2), can be used to exploit these same properties of alcohol, and enables considerable numbers of determinations to proceed simultaneously with a minimum of attention, so that alcohol determinations can be readily combined with other estimations on the same blood samples.

Winnick (3) has described the application of Conway units to the determination of alcohol in blood, the principle employed being essentially that of evaporation of the blood in the outer compartment and absorption of alcohol in the center compartment by means of dichromate and sulfuric acid, the excess dichromate being finally determined iodometrically. As in the Widmark (4) method, heat must be applied in order to secure rapid absorption, and this in turn necessitates the use of a special fixative. In the present method, saturated potassium carbonate is employed in the outer compartment to assist the expulsion of alcohol, and ready absorption at room temperature is secured by the use of alkaline permanganate in the center chamber. This reagent will absorb free acetone even more readily than alcohol, and in fact the method here described could be used with very slight modification for the determination of free acetone if desired. Free acetone in quantity in the blood is, however, of rare occurrence and the writer has not experienced trouble from this source following experimental administration of alcohol. By a modification mentioned later, alcohol and acetone may be determined in the presence of each other.

## *Reagents—*

1. Approximately 0.04 M potassium permanganate in 3 N potassium hydroxide. 0.63 gm. of potassium permanganate, analytic reagent, is added to 100 ml. of a solution containing 17 gm. of potassium hydroxide, analytic reagent, in distilled water made up to 100 ml. and stored in an amber glass bottle. The bottle is stoppered and shaken at intervals until the permanganate is totally dissolved. The entry of carbon dioxide to the

alkaline solution should be kept to a minimum during preparation and handling.

2. 10 per cent, weight by volume, barium chloride ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in water. This solution should be kept well stoppered when not in use.

3. A saturated solution of potassium carbonate in water.

4. 0.04 M solution of thiourea in water. This solution is standardized as described later, and is diluted for immediate use twenty-five times to give 0.0016 M strength. 250 ml. of solution contain 0.76 gm. of thiourea.

5. Commercial water glass, very slightly diluted with water to facilitate application as a lid fixative.

### *Procedure*

A number of Pyrex glass Conway units (cleaned as described below), sufficient to include estimations and two blanks, is laid out on the bench and slightly tilted on strips of cardboard 1 to 2 mm. thick. The lids are in position but not smeared with fixative. Before assembly of the units, which are best stored in an inverted position on metal trays in a cupboard, both dish and lid should be inspected to insure the absence of dust, fluff, etc.

0.5 ml. of saturated potassium carbonate is pipetted into the outer compartment of each dish at the lowest level, and the lids are then smeared with fixative and lightly replaced. Into the center compartment of each dish 1 ml. of alkaline permanganate is pipetted carefully by means of a pipette of the Ostwald-Van Slyke pattern which insures very uniform pipetting throughout. The lids are rotated to fix them, and the units covered by a dark cloth until required.

To carry out the estimation, 0.1 ml. of blood is delivered into the outer compartment at the highest level, the lid is firmly sealed, and the dish rotated to mix blood and carbonate and then placed in a dark cupboard. The dish is ready for titration 2 hours later, though the general practice has been to allow 3 hours to insure complete absorption. The dishes may, if desired, be left overnight, the blanks being treated similarly. When blood is obtained from a skin prick, 1 per cent cetyltrimethylammonium bromide may be used for cleansing and disinfecting, with oxalate or fluoride as the anticoagulant. For the estimation of acetone, an absorption period of half an hour was found adequate.

*Titration*—In order to secure the maximum sensitivity and convenience for operations in Conway units, the principle of titrating over the stage of permanganate to manganate was adopted, this first step of reduction being that produced by the alcohol during absorption, so long as the capacity of the permanganate (0.300 to 0.350 mg. of alcohol) was not exceeded. A reducing agent had to be found which would reduce the permanganate

very rapidly to the stage of manganate and very slowly beyond. In order to overcome the difficulty of detecting the end-point in the presence of the intense green color of the manganate, barium chloride was used to precipitate manganate as the insoluble barium salt. Several substances were found to possess the requisite properties, including potassium iodide, potassium thiocyanate, potassium formate,  $\alpha,\beta$ -unsaturated acids,  $\alpha$ -hydroxy acids, sodium thiosulfate, and thiourea. Of these, only fumaric acid, sodium thiosulfate, and thiourea were found to react sufficiently rapidly to give sharp end-points. The fumaric acid solution, while satisfactory when fresh, deteriorated very rapidly and was useless after 24 hours. Both thiosulfate and thiourea have been used in practice, but, for reasons given later, thiourea is preferable. Suitable strengths of these solutions were found to be 0.002 M sodium thiosulfate and 0.0016 M thiourea, for use with the standard Conway units of the center compartment capacity to about 4 ml., with a 2 ml. micro burette (with reservoir) graduated in divisions of 0.02 ml., capable of being read by eye to 0.002 ml. Preliminary tests showed that the amount of reducing agent required was proportional to the amount of permanganate present, over the range of the method. Such an experiment may be carried out by diluting the permanganate with 3 N potassium hydroxide. Since every specimen of potassium hydroxide handled by the writer has possessed the power of causing some reduction of permanganate to manganate, it was found necessary, before carrying out dilutions, to add permanganate in small amounts to the potassium hydroxide solution until a faint pink tinge remained, and to make a correction for the small amount of reagent required to reduce the excess. The data presented below indicate that the amount of available oxygen consumed by the alcohol is also proportional to the amount present, over the range of alcohol concentrations to which the method is applicable (0 to 300 mg. per cent with 0.1 ml. samples, 0 to 600 mg. per cent with 0.05 ml.).

In order to perform the titrations 0.2 ml. of 10 per cent barium chloride is added to the center compartment and the contents gently stirred by means of a glass rod drawn down to 1 to 1.5 mm. for a length of 6 cm., and bent, 10 to 15 mm. from the end, to 135°. The stirring is continued during dropwise addition of the reducing agent, till the last trace of pink color has disappeared. Fractional drops may be carried on the rod. The end-point is recognized by the apparent sudden change of color of the precipitated barium manganate viewed through the solution, from steel-blue to dark green.

*Calculation of Results*—The formula applicable is  $(B - E) \times C = \text{mg. of alcohol}$ , or  $(B - E) \times 1000C = \text{mg. per cent of alcohol for 0.1 ml. of specimen}$ , where  $B$  = the blank titration in ml.,  $E$  = estimation in ml.,

and  $C$  = a factor determined experimentally on alcohol solutions of known strength.  $C$  will then be the number of mg. of alcohol to which 1 ml. of the reducing agent, *e.g.* thiourea, is equivalent. The thiourea is best standardized directly against known amounts of alcohol in the Conway units. A suitable standard solution may be made by delivering 1.000 ml. of pure alcohol into a 500 ml. volumetric flask and diluting to the mark with water, or by making a solution of twice this strength and employing a range of dilutions. In the former case, the solution contains 159 mg. per cent of alcohol and 0.1 ml.  $\equiv$  0.159 mg. of alcohol.

As a rigorous test of the method under laboratory conditions, the following experiment was carried out. On 3 separate days, alcohol solutions of fixed strengths were made up and estimated, and on each day fresh dilutions of a stock solution of thiourea were used for titration. The dilute thiourea was ascertained to titrate 1 ml. for  $0.177 \pm 0.0015$  mg. of alcohol. The results include all accumulated errors of manipulation arising from dilution of alcohol solutions, dilution of thiourea, measurement and introduction of samples, pipetting of permanganate, and titration error, as well as possible lack of uniformity in cleanliness of dishes, contamination with atmospheric dust, etc.

The results obtained, which are shown in Table I, indicate that, over a considerable range of alcohol concentrations, the method gave good results. It should be stressed that the figures shown are for individual estimations, not the mean of two or more, and it appears that the over-all error, assuming the alcohol dilutions to have been made correctly, was of the order of  $\pm 0.003$  mg. for a single estimation. When mean values are calculated, the agreement between experimental and calculated figures is good.

In Table II are given some values obtained on oxalated rat blood to which alcohol was added in known amounts. This method of producing different alcohol concentrations was not completely satisfactory, however, and, as shown in Table III, the results obtained by estimating duplicate blood samples taken from animals to which alcohol had been administered gave close agreement between duplicates. The amount of volatile reducing substance in the blood of untreated animals is also seen to be negligible.

*Notes on Method and Reagents*—The dishes used must be chemically clean. If this is not the case, the fact will be readily detected by the failure of the permanganate to spread at once evenly over the glass surface of the center compartment, and such a dish should be rejected.

The fixative used, water glass, was adopted in preference to fixatives embodying organic substances which might cause reduction of permanganate if a filament is drawn out during removal of the lid for titration. It is easily and completely removed by water and leaves no contaminating

substance on the glass. It should not be allowed to remain in place more than 24 hours, and must be completely removed before acid is used.

TABLE I  
*Determination of Alcohol Solutions*

Date	Thiourea used	Alcohol found	Alcohol calculated
1947	ml.	mg.	mg.
Sept. 11	1.329	0.235	0.239
	0.905 <sup>a</sup>	0.160	0.159
	0.607	0.107	0.111
	0.377	0.067	0.064
Sept. 12	1.360	0.241	0.239
	0.884	0.156	0.159
	0.640	0.113	0.111
	0.320	0.057	0.064
Sept. 16	1.357	0.240	0.239
	0.901	0.159	0.159
	0.633	0.112	0.111
	0.350	0.062	0.064
Mean calculated from 3 determinations on 4 solutions		0.239	0.239
		0.158	0.159
		0.110	0.111
		0.062	0.064

1 ml. of thiourea solution =  $0.177 \pm 0.0015$  mg. of ethyl alcohol; error,  $\pm 0.003$  mg. for a single determination.

TABLE II  
*Recovery of Alcohol Added to Rat Blood in Vitro*

Alcohol calculated	Alcohol found	
	1st series	2nd series
mg.	mg.	mg.
0.145	0.150	0.150
0.099	0.097	0.107
0.076	0.076	0.069
0.051	0.050	0.045

The following routine procedure has proved satisfactory for cleansing the units. As soon as possible after completion of titration, the dishes and lids are thoroughly washed under the tap. The lids may at once be dried with a towel and put away after removal of fluff. The dishes are soaked in dilute hydrochloric acid to remove barium carbonate, then rinsed and filled with dichromate-sulfuric acid cleaning mixture. After standing



1 to 2 hours, they are rinsed with tap water and distilled water, stacked in an inverted position on metal trays, and dried in the oven. They are then stored inverted in a cupboard free from dust until required.

TABLE III  
*Determinations on Blood of Alcohol-Treated and Untreated Animals*

	Experiment 1	Experiment 2	Experiment 3
	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
Blood from rats treated with ethyl alcohol by inhalation; 0.03 ml. specimens from tail measured in specially calibrated capillary pipettes; duplicate determinations	2.308*	2.300*	2.225*
	2.290*	2.304*	2.252*
	1.912	2.050	1.740
	1.892	2.048	1.763
	1.884	1.982	1.780
	1.900	1.984	1.812
	1.940	1.898	1.930
	1.920	1.920	1.932
		1.980	1.812
		2.000	1.782
	Blank	Estimation†	Reducing substances calculated as acetone
	<i>ml.</i>	<i>ml.</i>	<i>mg. per cent</i>
Blood from fasting untreated rabbits; 0.1 ml specimens from marginal ear vein	2.580	2.590	
	2.580	2.500	6
	2.351	2.332	2
	2.351	2.330	2
	2.348	2.305	3
	2.348	2.285	5
	2.316	2.266	4
	2.316	2.252	5
	2.172	2.110	4
	2.172	2.092	6
	1.974	1.977	

1 ml. of 0.002 M thiosulfate used = 0.147 mg. of alcohol or 0.078 mg. of acetone.

\* Blank.

† Each figure represents blood from a separate animal, the animals being usually bled in pairs.

By treatment of the alkaline permanganate solution with one-fifth of its volume of 10 per cent barium acetate and centrifugation of the barium manganate, a permanganate solution is obtained which is free of manganate and can be titrated under conditions similar to those used in the estimations. Since the available oxygen involved in the reduction of permanganate to manganate is then exactly one-fifth of the total avail-

able oxygen determined iodometrically, it is thus possible to establish the absolute reducing power of the solutions used for reduction and to draw conclusions about the oxidation products. Thiosulfate apparently does not yield a single oxidation product. Calculation of the number of oxygen equivalents per mole consumed in the oxidation has given the value 7.3 on several occasions, even when freshly prepared thiosulfate solutions were tested. Since oxidation to sulfate involves uptake of 8 equivalents, this indicates formation of another oxidation product involving fewer equivalents per mole. The explanation does not apparently lie in the preliminary formation of a lower oxidation product, *e.g.* dithionate, with slow subsequent oxidation to sulfate, since, if a blank titration is interrupted for half an hour at the half way point and then completed, the end-point is unchanged. Rather, it appears that the oxidation takes two courses from the start. An explanation might be found in the possibility of resonance in the thiosulfate ion, so that the linkage between the sulfur atoms could behave both as a coordinate and covalent bond, with the formation of two distinct oxidation products. In the case of thiourea, the number of equivalents of oxygen consumed per mole was found to be close to 11, an unexpectedly high figure which suggests cyanate as the final oxidation product. As thiourea solutions were found to keep well, and, moreover, since this reagent is relatively insensitive to considerable alteration in the titration conditions, such as a gross excess of barium chloride, thiourea appears the preferable reducing agent..

Comparison of the empirical factor for alcohol with the reducing power of the solution also permits calculation of the number of equivalents of oxygen taken up by alcohol during oxidation. This shows that, while the main product is certainly acetate (with uptake of 4 equivalents per mole of alcohol), the oxygen consumption is 7 to 9 per cent more than that calculated for this oxidation, probably the consequence of slight enolization of the acetaldehyde presumably formed as the intermediate product. The conditions are thus quite different from those of Friedemann and Klaas (5), who found oxalate as the product of oxidation of ethyl alcohol by boiling alkaline permanganate.

The use of potassium carbonate as an agent to increase the vapor tension of alcohol solutions and so increase absorption in Conway units at room temperature is referred to in a very brief notice by Ryan *et al.* (6), apparently in conjunction with Winnick's (3) absorbent, the statement being made that absorption was "almost complete" in 1 hour at room temperature. When the writer was developing the present method some 3 years ago, it appeared to him that the reaction with dichromate and sulfuric acid was rather slow for the purpose of a microdiffusion method designed to operate at room temperature, and this led to the use of alkaline perman-

ganate. Winnick's absorbent has the advantage of not being sensitive to acetone. The acetone-absorbing property of alkaline permanganate has not in the writer's experience proved to be a disadvantage under the conditions of experimental administration of alcohol, and, owing to the rarity of occurrence of free acetone in considerable quantity in the blood, errors due to this substance have been ignored in some methods; *e.g.*, that of Widmark (4).

The method described in this paper can, if necessary, be adapted to the estimation of alcohol in the presence of acetone by substituting for the saturated potassium carbonate solution a buffered solution of potassium sulfite made as follows: 5 gm. of potassium hydroxide are dissolved in 12 ml. of water and cooled, and 11 gm. of potassium metabisulfite are added in small quantities. Finally water is added dropwise until the added salt just dissolves. The pH should then be close to 8. This solution, which keeps for about a week in a stoppered flask, has the property of permitting alcohol to diffuse completely in a 2 hour period, although retaining 93 to 95 per cent of the acetone up to 40 mg. per cent. During the absorption period, a very slight but perceptible reduction of the permanganate in the blank dish takes place (amounting to 0.03 to 0.05 ml. in the titration) due to absorption of sulfur dioxide. The necessity which thus arises of completing the titration within a period of approximately 3 hours from the introduction of the sulfite detracts somewhat from the convenience of the method in this form. Acetone has approximately twice the reducing power of alcohol towards alkaline permanganate. Microdiffusion methods for acetone have been given by Werch (7) and Winnick (8), depending on absorption in the Denigès reagent and bisulfite respectively.

The alkaline permanganate solution deteriorates slowly in the bottle, but, if well protected from the atmosphere, can be kept for 2 to 3 weeks. It should be discarded when the alcohol-oxidizing capacity falls appreciably below 0.300 mg. per ml. (blank titration, 1.7 ml. of 0.0016 M thiourea). When the oxidizing capacity is nearly exhausted by the alcohol present in the unit (titration of residual permanganate <0.15 ml. of 0.0016 M thiourea), formation of oxides of manganese may commence, and this invalidates the calculation based on reduction to manganate. During titration, the removal of manganate ions by barium effectively prevents such further reduction.

Carbon dioxide must not be allowed to gain entry to the potassium hydroxide in large amounts; otherwise this action of the barium is disturbed, but the small amount of carbon dioxide absorbed from the air during titration does not interfere. The precipitated barium manganate is stable in the strongly alkaline solutions employed, but thick crusts of

this substance should not be allowed to accumulate on the stirring rod, as permanganate can be formed on exposure to the air, due to absorption of carbon dioxide.

The alkaline permanganate should not be exposed to an atmosphere contaminated by organic vapors (especially alcohol), dust, or tobacco smoke. The barium chloride also should be protected from alcohol vapor, as appreciable amounts may be absorbed from the air.

Finally it may be mentioned that if alcohol, or better, acetone is introduced into the outer compartment of a Conway unit prepared as described with potassium carbonate as the expelling agent, the change of color of the permanganate solution due to reduction can be easily detected by the eye by comparison with a blank dish after quite a short time. This affords a vivid visual demonstration of the microdiffusion principle in operation.

#### SUMMARY

1. A method of determining ethyl alcohol in blood specimens of 0.1 ml. is described, the principle of microdiffusion being employed.

2. By the use of potassium carbonate as an alcohol-expelling agent and alkaline permanganate for absorption, the Conway units can be conveniently operated at room temperature.

3. A method of titrating alkaline permanganate is described, based on reduction to manganate by thiourea in the presence of barium ions.

4. By the employment of a buffered solution of potassium sulfite to expel alcohol, interference by acetone can, if necessary, be largely eliminated.

The writer desires to express appreciation of the interest shown by Professor F. L. Golla. He is also indebted to Mrs. R. K. Carpenter and Mr. D. Roberts for technical assistance, and to the Monthly Bulletin Research Fund of the British Society for the Study of Addiction, which supported the work.

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## TRYPTOPHAN METABOLISM

### XII. TRYPTOPHAN, KYNURENINE, AND RELATED COMPOUNDS AS PRECURSORS OF NICOTINIC ACID\*

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(Received for publication, July 11, 1949)

It is well established that tryptophan promotes an increase in the urinary output of nicotinic acid derivatives (1, 2) and that a deficiency of niacin in the diet can be met or offset by an increased intake of tryptophan (3). The question of whether tryptophan is actually itself a precursor of niacin (2) or functions only indirectly to stimulate its production now appears to have been resolved in favor of the former view. Direct evidence is had in the recent demonstration that the *N*<sup>1</sup>-methylnicotinamide excreted after tryptophan-3-C<sup>14</sup> is fed contains the isotope in its carboxyl group (4).

The purpose of the present communication is to record the results of tests begun some months ago to determine comparatively the capacities of D- and L-tryptophan, indole derivatives, and certain metabolites of tryptophan, particularly kynurenine, to produce methylnicotinamide in the rat. So far as we are aware, no direct comparisons of D- and L-tryptophan have previously been made. During the early course of the tests, kynurenine, kynurenic acid, and xanthurenic acid were said (5) to have produced no extra urinary *N*<sup>1</sup>-methylnicotinamide in the rat. It was therefore assumed that the synthesis of niacin from tryptophan could not proceed via kynurenine (5). In tests made elsewhere with mutant strains of *Neurospora*, quite the opposite conclusion was reached (6). The data recorded in this paper afford direct evidence that, even when injected subcutaneously, kynurenine does promote the excretion of extra methylnicotinamide in the rat. Together with recently published circumstantial evidence (4, 7, 8), the observations leave little doubt that kynurenine is a normal intermediate in the production of niacin from tryptophan in this animal.

#### EXPERIMENTAL

The DL-tryptophan used in these studies was a synthetic product.<sup>1</sup> It was acetylated and resolved, essentially as directed by Berg (9). The

\* The data in this paper were taken largely from a thesis submitted by Mr. Kallio in June, 1948, in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry, in the Graduate College of the State University of Iowa.

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<sup>1</sup> We are pleased to acknowledge our indebtedness to Dr. L. S. Roehm and The Dow Chemical Company for a generous gift of synthetic tryptophan.

optical rotations of the acetyl-L- and acetyl-D-tryptophan and the L- and D-tryptophan obtained are recorded in Table I.

The L-kynurenine and the kynurenic acid were isolated from the urines of rabbits given 4 gm. doses of L-tryptophan, usually by stomach tube but in a few instances by subcutaneous injection. 24 hours later the urine remaining in the bladder was obtained by abdominal pressure and added to the urine voided. The combined sample was made acid to Congo red with 5 per cent sulfuric acid, to precipitate the kynurenic acid. After standing at least overnight in the refrigerator, the kynurenic acid was filtered off. Enough 50 per cent sulfuric acid was added to the filtrate to raise its sulfuric acid concentration to 5 per cent by volume. It was then mixed with an equal volume of 10 per cent mercuric sulfate in 5 per cent (by vol-

TABLE I  
*Specific Rotations of Optically Active Compounds Tested*

Substance	[ $\alpha$ ] <sub>D</sub>		Concentration per 100 cc. solution		Solvent	Temperature	Bibliographic reference No.
	Found	Recorded in reference	As read	Recorded in reference			
	degrees	degrees	gm.	gm		°C.	
Acetyl-L-tryptophan	+26.0	+26.1	1.0	0.5	MeOH	20	(9)
Acetyl-D-tryptophan	-26.2	-26.2	1.0	0.5	"	20	(9)
L-Tryptophan	-32.2	-32.1	0.5	0.5	H <sub>2</sub> O	20	(9)
D-Tryptophan	+32.1	+31.6	0.5	0.5	"	20	(9)
L-Kynurenine sulfate	+10.2	+10.0	0.5	2.0	"	30	*
"		+10.7		0.87	"	16	(10)
"		+7.8		Unstated	"	Unstated	(11)

\* Rowe, V. K., and Berg, C. P., unpublished data.

ume) sulfuric acid and set aside for 48 hours at 0°. The brown mercuric sulfate complex of kynurenine was removed by centrifugation, washed twice with 5 per cent sulfuric acid solution, twice with water, then suspended in water and decomposed with hydrogen sulfide. The mercuric sulfide was filtered off and the filtrate concentrated *in vacuo* to about 25 to 15 cc. Absolute alcohol was added to make the mixture 85 to 90 per cent alcoholic. The long, needle-shaped, buff crystals of kynurenine sulfate obtained after 24 hours in the refrigerator appeared to be identical with those in the photomicrograph published by Kotake and Iwao (10). Further fractionation of the mother liquors failed to yield appreciable additional amounts of the product.

Several recrystallizations from 75 per cent ethyl alcohol produced less highly pigmented crystals, having the specific rotation indicated in Table I. They responded to the Kikkawa test for kynurenine (12), but not to the

Hopkins-Cole test. When heated in a capillary tube, they began to darken around 170° and melted, with blackening, at 194–195°. Corresponding temperatures recorded by Butenandt and his associates (11) are 165° and 194°. The nitrogen content (by Kjeldahl) was 8.71 per cent, in good agreement with the 8.80 per cent recorded by Butenandt *et al.*, but agreeing less well with the 9.15 per cent calculated from the anhydrous formula which they assign to kynurenine sulfate.

The precipitated kynurenic acid was partially purified as described by Berg (13), after which it melted at 262–263° (uncorrected). Values recorded in the literature vary widely, usually between 257° and 278° (13).

The yields of L-kynurenine per 4 gm. dosage of tryptophan ranged from 0.0556 to 1.66 gm. and averaged 0.935 gm. in the eleven rabbits employed. The yields of kynurenic acid ranged from 0.886 to 1.878 gm. and averaged 1.369 gm.

The xanthurenic acid<sup>2</sup> melted at 289–290°, thus comparing favorably with the melting point of 288° reported by Lepkovsky, Roboz, and Haagen-Smit (14).

The indole-3-pyruvic acid was prepared in the usual way (15, 16) by condensing indole-3-aldehyde with hippuric acid in the presence of acetic anhydride and anhydrous sodium acetate, and hydrolyzing the acetylated azlactone with alkali. The indole aldehyde was made from indole by a modified Reimer-Tiemann reaction (17). The purified indolepyruvic acid was light tan in color. It softened at 204° and melted at 210–211°. The literature records 212° as the melting point (16). The *p*-nitrophenylhydrazine derivative melted at 153°, as compared with 153–154° recorded by others (15, 16).

The indole-3-acetic and indole-3-propionic acids were purchased from the Eastman Kodak Company. They melted at 167–168° and 132–133°, respectively.

The compounds listed were each tested for their capacity to promote niacin synthesis by injecting them subcutaneously into adult female rats maintained on a diet deficient in tryptophan and nicotinic acid and determining the urinary excretion of *N*<sup>1</sup>-methylnicotinamide during the following 24 hours. The rats were of the Sprague-Dawley strain; they averaged 300 gm. in weight, and were housed individually in cages placed in funnels fitted with screens to retain the feces and tubes to collect the urine. The tryptophan-deficient diet was fed *ad libitum*. Per 100 gm., it contained acid-hydrolyzed casein 17.7, L-cystine 0.3, glucose 15.0, starch 37.0, Vream<sup>3</sup> 19.0, cod liver oil 5.0, salt mixture (18) 4.0, and agar 2.0 gm.

<sup>2</sup> This was made available to us through the courtesy of Dr. Richard H. Barnes of Sharp and Dohme, Inc.

<sup>3</sup> Vream is a hydrogenated mixture of animal and vegetable fat marketed by Swift and Company.



A mixture of the B complex vitamins was also fed separately each day in pellet form, except during the 24 hour periods in which the urine was collected. The mixture contained no nicotinic acid. Per day, it provided thiamine hydrochloride 40, riboflavin 60, and pyridoxine hydrochloride 60  $\gamma$ ; calcium pantothenate 0.2, *p*-aminobenzoic acid 2.0, inositol 2.0, choline hydrochloride 20.0, corn syrup 50, and starch 100 mg. A minimum of 7 days was allowed after transfer to the experimental diet before the injections were begun, and 3 days were allowed to intervene between injection periods to insure the return of the *N*<sup>1</sup>-methylnicotinamide excretion to the basal level.

To determine its capacity to promote *N*<sup>1</sup>-methylnicotinamide excretion, each of the compounds, but one, was tested in a series of twelve rats. These were separated into three groups of four animals each. Each of the rats in the first group was injected with the test compound in an amount equivalent to 100 mg. of tryptophan and each in the second group with 100 mg. of L-tryptophan. The rats in the third group served as basal controls and received no injections. In the two succeeding test periods the order was changed so that by the end of the third period each rat had served as an experimental animal, as an L-tryptophan control, and as a basal control. In some of the test series, one or more of the rats developed sterile abscesses at the site of the injections and were therefore discarded before all of the tests were completed. In the series of tests on xanthurenic acid, the supply of the compound sufficed for only eight animals. All urines were analyzed at least in duplicate for *N*<sup>1</sup>-methylnicotinamide, essentially as directed by Huff and Perlzweig (19). Under the conditions employed, none of the compounds tested was found to interfere with the determination.

#### DISCUSSION

The results of the tests are summarized in Table II. On all of the regimens, marked variations in output of *N*<sup>1</sup>-methylnicotinamide occurred. Comparable divergence in data from other laboratories (20) seems to indicate that wide variations may be inherent in this type of study. Rotation of the animals to test each on all three regimens in the series should have compensated for differences in their synthetic capacities. In spite of the deviations on each regimen, comparison of the average net excretion after injection of the compound being tested with the average net excretion after the injection of L-tryptophan seems to afford a fairly valid, though rough, estimate of the relative utility of the compound for *N*<sup>1</sup>-methylnicotinamide production.

In Series I, pyridoxine was added to the solution of tryptophan injected to determine whether this would markedly accelerate the methylnicotinamide excretion. It has been both claimed (5, 21, 20) and denied

TABLE II

*N*<sup>1</sup>-Methylnicotinamide Excretion in Urine of Rats in 24 Hours after Subcutaneous Injection of Test Substance in Dosages Equivalent to 100 Mg. of Tryptophan

Series No.	Substance injected	No. of rats	<i>N</i> <sup>1</sup> -Methylnicotinamide excretion		Relative availability of test substance for <i>N</i> <sup>1</sup> -methylnicotinamide production*
			Average	Range	
			γ	γ	per cent
I	L-Tryptophan (+50 γ pyridoxine HCl)	11	1290	820-1925	115
	L-Tryptophan	11	1128	783-1560	
	None	12	52	5- 181	
II	D-Tryptophan	12	1115	216-2405	67
	L-Tryptophan	10	1612	290-2500	
	None	12	56	8- 108	
III	DL-Tryptophan	9	1528	806-2500	95
	L-Tryptophan	11	1605	600-2810	
	None	10	150	62- 252	
IV	Acetyl-L-Tryptophan	12	509	36-1278	38
	L-Tryptophan	12	1204	250-3035	
	None	12	87	15- 185	
V	Acetyl-D-Tryptophan	12	84	41- 176	1
	L-Tryptophan	12	1456	176-2652	
	None	12	64	15- 106	
VI	L-Kynurenine sulfate	12	526	60-1520	40
	L-Tryptophan	11	1202	282-2890	
	None	13	77	21- 150	
VII	Kynurenic acid	11	46	17- 85	0
	L-Tryptophan	11	850	240-1680	
	None	11	43	17- 145	
VIII	Xanthurenic acid	8	82	34- 104	2
	L-Tryptophan	8	1058	788-1569	
	None	8	62	24- 124	
IX	Indole-3-pyruvic acid	11	1200	174-1852	69
	L-Tryptophan	11	1712	224-2850	
	None	12	76	7- 213	
X	Indole-3-propionic acid	12	190	40- 250	1
	L-Tryptophan	12	1757	650-2960	
	None	12	166	48- 330	
XI	Indole-3-acetic acid	12	109	62- 160	1
	L-Tryptophan	12	2038	835-2640	
	None	12	86	38- 186	

\* (Average excretion after injection of test substance) -  
 (average control excretion (no injection))  
 (Average excretion after L-tryptophan injection) -  
 (average control excretion (no injection)) × 100.

(22) that a deficiency in pyridoxine interferes appreciably with the production of methylnicotinamide from tryptophan. It seemed barely possible that, if pyridoxine were required in the process, the injection of tryptophan might impose an extra demand for it sufficient to overwhelm the capacity of the tissues to mobilize it and thus induce a deficiency. The increase of 15 per cent noted in the output of methylnicotinamide is probably not sufficiently marked to justify making any broad deductions.

D-Tryptophan (Series II) appears to be well utilized for *N*<sup>1</sup>-methylnicotinamide production. The fact that indolepyruvic acid (Series IX) promotes a similar output correlates well with earlier observations that both D-tryptophan and indolepyruvic acid can be used about as well as L-tryptophan for growth in the rat (23, 24), presumably because they can be converted into this isomer. Since they are not also as readily converted into kynurenic acid when administered in amounts larger than those which suffice for growth (25), it seems reasonable to assume that their conversion into L-tryptophan must be limited. Although D-tryptophan yields D-kynurenine in the rabbit (26, 10) and presumably also in the rat (25), this form of kynurenine apparently cannot be converted into kynurenic acid (26, 25) by these species, or into niacin by *Neurospora crassa* (6).

Comparative tests leave little doubt that L-kynurenine sulfate (Series VI) can induce niacin synthesis in the rat. This finding seems especially significant. It confirms previous observations with *Neurospora crassa* (6) and lends support to the circumstantial evidence afforded by the conversion of tryptophan- $\beta$ -C<sup>14</sup> into kynurenine (8), and of tryptophan-3-C<sup>14</sup> into *N*<sup>1</sup>-methylnicotinamide (4), presumably via kynurenine. The rather wide individual differences noted in our tests with L-kynurenine and the limited availability of this product to the research worker suggest that the negative findings reported by others (5, 27), in support of which they presented no data, may have been based on a very limited number of tests. On the other hand, our data on *N*<sup>1</sup>-methylnicotinamide excretion after the injection of kynurenic acid (Series VII) and xanthurenic acid (Series VIII) support the comment (5) that neither of these increases niacin production. Our data on indolepropionic acid (Series X) and on indoleacetic acid (Series XI) also show little or no increase in *N*<sup>1</sup>-methylnicotinamide above the output under basal conditions. Again the literature refers to unpublished data said to afford similar evidence (28).

Acetylation retarded considerably the capacity of L-tryptophan to promote niacin synthesis (Series IV) and made D-tryptophan completely unavailable for this purpose (Series V). These results agree with previous observations that, although acetyl-L-tryptophan can be utilized as well for growth as L-tryptophan (23, 24), it yields much less kynurenic acid (23). Acetyl-D-tryptophan, on the other hand, is quite useless either for growth

(23, 24) or as a source of kynurenic acid (23). Since L-kynurenine is unable to replace tryptophan for growth in the rat (29), the acetyl-L-tryptophan so used must undergo deacetylation, without cleavage of the indole ring. Such an assumption would be unnecessary to account for the production of kynurenine or its conversion to *N*<sup>1</sup>-methylnicotinamide. The fact that acetyl-D-tryptophan cannot replace L-tryptophan for growth in the rat, as D-tryptophan can, argues against its deacetylation before ring cleavage, but does not preclude the possibility that it might first be converted to acetyl-D-kynurenine, then be deacetylated. The capacity of D-kynurenine to produce niacin in the animal organism has not been determined. It is not converted to kynurenic acid in the animal (26, 25), nor does it appear to serve as a niacin precursor in *Neurospora crassa* (6).

The less efficient production of *N*<sup>1</sup>-methylnicotinamide from L-kynurenine (Series VI) than from L-tryptophan is not necessarily out of harmony with the view that *N*<sup>1</sup>-methylnicotinamide is produced via L-kynurenine. It is quite possible that the concentration of kynurenine afforded by a single large injection might overwhelm the capacity of the organism to convert it to nicotinic acid and result in the shunting of a considerable portion of it to a more readily available metabolic outlet. Injection of an equivalent amount of L-tryptophan, on the other hand, might be expected to provide L-kynurenine at a rate better suited to nicotinic acid synthesis. The fact that *N*<sup>1</sup>-methylnicotinamide is produced from L-kynurenine, an intermediate metabolite of L-tryptophan quite incapable of supporting growth, emphasizes a point often overlooked, that evidence based on metabolite excretion does not always provide a safe criterion as to relative utility for growth.

Though the compounds used in these studies were injected subcutaneously in order to by-pass the intestines, the possibility that niacin may have been synthesized in part by the intestinal flora is nevertheless not completely excluded. In some animals at least one metabolite of tryptophan (kynurenic acid) can be eliminated via the bile and thus reach the intestinal lumen. It seems quite unlikely, however, that excretion by this route could have provided enough of any metabolite to have accounted for more than a small fraction of the increment in the *N*<sup>1</sup>-methylnicotinamide excretion noted after the subcutaneous injection of the substances most effective in producing it.

#### SUMMARY

Several compounds which bear a metabolic or structural relationship to L-tryptophan have been tested for their capacities to promote the synthesis of nicotinic acid in rats maintained on a diet deficient in tryptophan and nicotinic acid. To circumvent, as completely as possible, the action of

bacteria in the alimentary tract, the compounds were injected subcutaneously. The  $N^1$ -methylnicotinamide content of the following 24 hour urines was determined. To afford maximal control, urines were collected from each rat in each series of tests, (a) after the injection of the test substance, (b) after the injection of an equivalent amount of L-tryptophan, and (c) after no injection.

Comparisons with the net excretion after the injection of L-tryptophan, as a standard, indicated that the net outputs after the injections of D-tryptophan and indolepyruvic acid were about a third smaller, but after the injections of DL-tryptophan, only slightly less. Acetylation completely blocked the capacity of D-tryptophan to produce an increased output of methylnicotinamide and lowered that of L-tryptophan by more than half. Indolepropionic and indoleacetic acids and kynurenic and xanthurenic acids failed to promote appreciable nicotinic acid synthesis.

The plausibility of assuming that L-kynurenine is a normal intermediate in the production of nicotinic acid from L-tryptophan is indicated (a) by the relatively high output of  $N^1$ -methylnicotinamide induced by its injection, (b) by the fact that L-kynurenine can be produced from, but is quite incapable of being reconverted into, L-tryptophan, and (c) by the fact that, in addition to L-tryptophan and L-kynurenine, the only other compounds which promoted appreciable increments in the output of  $N^1$ -methylnicotinamide were those which could be converted into L-tryptophan, hence were potentially able also to produce L-kynurenine. The amount of extra  $N^1$ -methylnicotinamide excreted in the urine after the injection of L-kynurenine sulfate, though large, was less than half that excreted after the injection of an equivalent dose of L-tryptophan. How this lower output can be reconciled with the thesis that L-kynurenine is an intermediate, is discussed.

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# EVIDENCE FOR THE SPECIFICITY OF ESTERASE AND LIPASE BY THE USE OF THREE CHROMOGENIC SUBSTRATES\*

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Following the development of a histochemical method for demonstrating non-specific esterase (1) in tissue sections by enzymatic hydrolysis of  $\beta$ -naphthyl acetate, the possibility was explored of developing a similar method for lipase together with convenient colorimetric methods for measuring the activity of both enzymes in blood serum.

Interest in the enzymes which hydrolyze carboxylic esters stems from the discovery by Hanriot in 1896 (2) of an enzyme in serum and tissues which hydrolyzed monobutyrin. He called it "lipase," a term which was immediately challenged (3, 4) on the ground that the serum does not hydrolyze true fats and that the enzyme is an esterase which splits the esters of the lower fatty acids. This difference of opinion still prevails (5-7). Presumably the ideal substrates for lipase are the glycerides of long chain fatty acids and for esterase the simple esters of short chain carboxylic acids. But a considerable degree of overlapping in enzymatic hydrolysis by these two enzymes occurs, since both enzymes can split esters of intermediate sized chains (8). The most effective hydrolysis which each enzyme produces (9, 10) depends more on the length of the acid chain than on the type of alcohol to which it is attached. Since the enzymes present in liver, kidney, and serum hydrolyze lower fatty acid esters quite readily and higher ones poorly, the action involved is considered to be due to esterase. Extracts of pancreas hydrolyze both types well and, therefore, probably contain both esterase and lipase (11). In order to study these differences further, so as to determine more precisely the specificity of these two enzymes with regard to short and long chain fatty acid esters and their relationship to certain enzymatic inhibitors and activators, the  $\beta$ -naphthyl esters of acetic ( $C_2$ ), lauric ( $C_{12}$ ), and palmitic-stearic ( $C_{16}$ - $C_{18}$ ) acids were prepared as substrates. Solutions or emulsions of these substrates were

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incubated with tissue homogenates, and, after a period of incubation, an azo dye was produced by coupling the  $\beta$ -naphthol liberated by enzymatic hydrolysis with a tetrazonium salt (tetrazotized diorthoanisidine). This colored compound was extracted from the reaction mixture with ethyl acetate and measured colorimetrically.

#### *Preparation of Substrates*

*$\beta$ -Naphthyl Acetate*—This was prepared from  $\beta$ -naphthol, acetic anhydride, and dry pyridine<sup>1</sup> as previously described (1).

*$\beta$ -Naphthyl Laurate*—Lauric acid (46 gm., Eastman grade, m.p. 42–43°) and thionyl chloride (40 cc.) were heated in an open Erlenmeyer flask until the acid dissolved. Heating near the boiling point was continued for another 10 minutes. Suction was then applied through a one-hole rubber stopper, and the excess thionyl chloride was evaporated.  $\beta$ -Naphthol (29 gm.) was added in portions to the hot acid chloride, and, when addition was complete and the reaction had subsided, 20 cc. of dry pyridine were added and the mixture was heated at 80–90° for 15 minutes. Absolute ethanol was added cautiously to the hot solution until 400 cc. had been added. The solution was cooled in an ice bath, and the ester was collected with the aid of suction and washed with absolute methanol. The yield was 55 gm. (83 per cent); m.p. 58–60°.

$C_{22}H_{38}O_2$ . Calculated, C 80.70, H 9.48; found, C 81.03, H 9.18

*$\beta$ -Naphthyl Stearate*—Stearic acid (30 gm., Eastman, m.p. 65–70°) and 30 cc. of thionyl chloride were used to prepare the acid chloride by the procedure given for the laurate.  $\beta$ -Naphthol (14.5 gm.) was added in portions, followed by 15 cc. of dry pyridine, and the mixture was heated for 15 minutes. Absolute ethanol (350 cc.) was added cautiously, the hot solution was cooled in an ice bath, and the ester was collected and washed with absolute methanol. The yield was 38 gm. (93 per cent); m.p. 73–75° as reported (12).

Better enzymatic hydrolysis was observed with the naphthyl ester prepared from crude stearic acid<sup>2</sup> than with the pure stearate. The crude acids melted at 57–59° and the crude naphthyl esters at 49–52°. The experiments reported below were performed with this crude palmitate-stearate mixture.

<sup>1</sup> Pyridine was made anhydrous by refluxing over barium oxide for several days, followed by distillation from barium oxide.

<sup>2</sup> Purchased from the Howe and French Company, Boston. The material contains 40 per cent stearic acid, 50 per cent palmitic acid, 8 per cent oleic acid, and traces of linoleic and myristic acids.

### Method

Fresh tissue obtained from several species and tissue from humans secured post mortem were weighed wet, homogenized in a ground glass tube, and centrifuged at 1500 R.P.M. for a few minutes to remove the coarse particles. Various dilutions with tap water were made.

*Substrates*— $\beta$ -Naphthyl acetate (10 mg.) was dissolved in 2 cc. of acetone and added to 50 cc. of veronal buffer<sup>3</sup> at pH 7.4 and 48 cc. of water. A clear solution resulted. In the case of the more insoluble naphthyl laurate and naphthyl stearate-palmitate, 10 mg. of each were dissolved in 10 cc. of acetone and added through a submerged pipette into an agitated solution containing 50 cc. of veronal buffer<sup>3</sup> at pH 7.4, 2 cc. of 1 per cent Aerosol solution<sup>4</sup> (O.T. 345), and 38 cc. of water. A faintly cloudy solution was produced (0.1 mg. per cc). The very pure naphthyl stearate did not produce a stable emulsion, but tended to crystallize. Preliminary experiments showed that this concentration of the substrates was in excess of that required for maximal rate of hydrolysis.

### Procedure

1 cc. of the tissue homogenate and 6 cc. of the substrate solutions were incubated (a) for 20 minutes at room temperature when naphthyl acetate was used, (b) for 1 hour at 37° when naphthyl laurate was used, and (c) for 3 to 24 hours at 37° when naphthyl stearate-palmitate was used. This timing was adopted to keep the amount of pigment production within the range of the Klett colorimeter, because of the greater activity of esterase compared to lipase. At the end of the incubation period 1 cc. of a freshly prepared, chilled solution containing 4 mg. of a stabilized tetrazonium salt (in powder form)<sup>5</sup> was added and the tube shaken. After a few minutes, 1 cc. of 40 per cent trichloroacetic acid was added to facilitate extraction of the azo dye from the protein-containing solution. The mixture was shaken with 10 cc. of ethyl acetate and centrifuged for 10 minutes. The red component of the purple azo dye was more evident in ethyl acetate solution than when precipitated from water. The solution was measured

<sup>3</sup> Prepared according to Michaelis (13) by mixing 58.1 cc. of a solution of 10.3 gm. of sodium diethyl barbiturate in 500 cc. of distilled water, with 41.9 cc. of 0.1 N hydrochloric acid.

<sup>4</sup> Aerosol was used throughout most of this study because of its value as an emulsifier. However, it was found toward the end to be unnecessary and, in addition, to produce some inhibition of enzymatic hydrolysis. In the experiments with sodium taurocholate, Aerosol was not used.

<sup>5</sup> Contains 20 per cent tetrazotized diorthoanisidine, 5 per cent zinc chloride, and 20 per cent aluminum sulfate. Provided through the courtesy of Dr. E. R. Laughlin, E. I. du Pont de Nemours and Company, Inc., Boston, under the trade name Naphthanil Diazo Blue B.

in a Klett photoelectric colorimeter with a No. 540  $m\mu$  filter. All reactions were run in duplicate. The colorimeter readings were converted to mg. and micromoles of  $\beta$ -naphthol from a calibration curve obtained with naphthol under conditions stated above. The curve was linear between 0.005 and 0.05 mg. of naphthol. When the ethyl acetate extract was diluted 1:1, the curve was linear between 0.01 and 0.1 mg. of naphthol. When inhibitors or accelerators were added, care was taken to maintain a pH of 7.4. The tissue homogenates were incubated with the inhibitor or accelerator for 1 hour before the substrates were added.

### Results

*Comparison of Enzymatic Hydrolysis of Three Substrates by Liver, Kidney, and Pancreas in Six Species*—The results of several experiments with each substrate and tissue are given in Table I. Kidney, liver, and pancreas hydrolyzed the acetic acid ester to about the same extent in all the species studied, whereas pancreas hydrolyzed from 50 to 100 times as much of the stearic-palmitic acid esters as did kidney and liver. Pancreas hydrolyzed about 10 times as much lauric acid ester as did liver in four species, but only 2 to 3 times as much in man, and about the same amount in the guinea pig. Balls and Matlack (14) obtained similar results with other butyric and stearic esters and concluded that pancreas contains not only esterase, but an additional enzyme, lipase.

*Effect of Inhibitors and Accelerators on Enzymatic Hydrolysis of Three Substrates*—Five substances have been widely studied for their effects upon the esterolytic action of tissue extracts. These are eserine (15), sodium taurocholate (16), quinine (17), sodium arsenilate (18), and sodium fluoride (19). Because of the sensitivity of our technique for detecting enzymatic activity, we found that the concentrations of these agents which have been suggested to demonstrate their near maximal activity were inadequate. We have accordingly used higher concentrations. The reagents were placed in contact with the enzyme for an hour at room temperature, following which the buffered substrate was added and the incubation carried out for the time and temperature specified above. Before treating the liberated  $\beta$ -naphthol with the diazonium compound, the same quantity of the various inhibitors was added to the control tubes. This was found necessary because of a slight but constant alteration in the pigment in the presence of many of these inhibitors. The technique of measurement was then continued as described. All reactions were run in duplicate. The results are presented in Tables II to VI. Each agent is discussed separately below.

*Eserine*—Although this substance is noted primarily for its inhibitory effect in low concentrations ( $10^{-5}$  M) on cholinesterase, it has also been said

to affect the esterase activity of human serum upon methyl butyrate and tributyrin (5, 20, 21). Huggins and Lapidès (22), however, found no inhibition of the enzymatic hydrolysis of *p*-nitrophenyl propionate by eserine

TABLE I  
*Esterolytic Activities of Kidney, Liver, and Pancreas upon Acetate, Laurate, and Stearate Esters of  $\beta$ -Naphthol\**

Species	Experiment No.	$\beta$ -Naphthyl acetate			$\beta$ -Naphthyl laurate			$\beta$ -Naphthyl stearate-palmitate		
		Kidney	Liver	Pancreas	Kidney	Liver	Pancreas	Kidney	Liver	Pancreas
Mouse	1	4.80	5.42	4.59	0.46	0.25	1.11	0.00070	0.00093	0.174
	2	3.75	5.42	3.11	0.36	0.24	0.97	0.00067	0.00116	0.327
	3	4.17	4.38	5.42	0.39	0.22	1.60	0.00064	0.00093	0.605
Rat	1	5.63	4.58	3.13	0.27	0.17	1.47	0.00049	0.00087	0.325
	2	5.63	4.58	3.54	0.30	0.17	0.81	0.00046	0.00081	0.348
	3	5.42	7.92	8.55	0.29	0.35	2.78	0.00044	0.00197	0.325
	4	5.63	8.55	7.72	0.29	0.47	2.64	0.00044	0.00180	0.325
	5	3.86	4.38	5.63	2.23	0.13	2.42	0.00041	0.00087	0.186
Guinea pig	1	2.40	23.4	2.92	0.25	0.53	0.42	0.00020	0.00046	0.0417
	2	2.29	22.1	5.22	0.23	0.53	0.63	0.00017	0.00041	0.0556
	3	1.46	15.4	3.76	0.19	0.53	0.42	0.00020	0.00087	0.0695
Rabbit	1	2.50	7.10	19.6	0.13	0.50	5.28	0.00049	0.00162	0.348
	2	2.80	7.30	24.2	0.15	0.50	3.20	0.00125	0.00157	0.186
	3	2.29	12.1	10.4	0.19	1.03	3.20	0.00157	0.00464	0.870
	4	2.39	10.8	12.9	0.12	0.83	3.75	0.00104	0.00447	0.852
Dog	1	0.62	5.84	9.60	0.042	0.21	2.08	0.00032	0.00058	0.220
	2	0.62	6.68	10.2	0.045	0.26	2.22	0.00032	0.00110	0.220
	3	1.21	3.12	5.00	0.038	0.24	1.32	0.00023	0.00064	0.162
	4	1.21	3.12	6.46	0.038	0.22	1.95	0.00020	0.00058	0.220
	5	0.58	3.55	3.96	0.052	0.22	1.39	0.00096	0.00250	0.500
Man	1	0.29	1.08	1.04	0.013	0.35	0.24	0.00017	0.00099	0.0163
	2	1.00	2.88	3.68	0.029	0.37	1.15	0.00145	0.00366	0.0766
	3	0.46	2.04	3.75	0.01	0.14	0.62	0.00020	0.00168	0.0302
	4	0.50	1.35	4.17	0.01	0.25	0.77	0.00035	0.00105	0.0348
	5	0.62	2.08	3.23	0.01	0.25	0.45	0.00020	0.00064	0.0626

\* The data are given in micromoles of  $\beta$ -naphthol liberated in 1 hour by 1 mg. of tissue (wet weight). It was found that the amount of substrate hydrolyzed was directly proportional to the time of incubation and the concentration of the enzyme. Calculation of the number of micromoles of naphthol produced by enzymatic hydrolysis was made with the following equation.

$$\frac{\beta\text{-Naphthol (micromoles)}}{\text{per mg. wet tissue per hr.}} = \frac{\beta\text{-naphthol (mg.)} \times 1000}{\text{weight (mg.)} \times \text{time (hrs.)} \times \text{mol. weight of naphthol}}$$

in this concentration. Similar observations were made by us with  $\beta$ -naphthyl acetate as a substrate. Eserine sulfate in  $3.5 \times 10^{-3}$  M concentration showed a small inhibitory effect on the hydrolysis of all three substrates by rat tissues, a somewhat greater effect by dog tissues, and a

very significant effect, especially with the acetic ester, by human tissues (Table II). The significance of this species difference is not clear.

*Sodium Taurocholate*—This compound at a concentration of  $2 \times 10^{-2}$  M was reported by Willstätter and Memmen (16) to have an accelerating effect upon the hydrolysis of methyl butyrate and triacetin by pancreas. More than the detergent action was involved in its ability to activate the pancreatic enzyme. This is supported by the report of Gomori that sodium taurocholate increased the intensity of pancreatic staining in his

TABLE II

*Effect of Eserine ( $3.5 \times 10^{-3}$  M) upon Esterolytic Activities of Kidney, Liver, and Pancreatic Extracts with Three Substrates\**

Species	Tissue	Inhibition of hydrolysis† of		
		$\beta$ -Naphthyl acetate	$\beta$ -Naphthyl laurate	$\beta$ -Naphthyl stearate-palmitate
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Rat	Kidney	12	30 (4)	25
	Liver	45	24 (4)	29
	Pancreas	70	3	23 (5)
Dog	Kidney	59 (4)	51	47
	Liver	61 (4)	50	40
	Pancreas	51 (5)	0†	57
Man	Kidney	86 (4)	67 (5)	53
	Liver	84	0†(5)	4
	Pancreas	83	46 (7)	65

\* The inhibitor remained in contact with the enzyme for 1 hour prior to incubation with the substrate solution. The percentages recorded represent the averages of three experiments run in duplicate. When more than three were performed, the number is indicated in parentheses.

† The esterolysis of each substrate by tissue was taken to be 100 per cent and the results in the presence of eserine are expressed as a ratio (per cent) in each experiment.

‡ Indicates acceleration of activity that was visible grossly.

histochemical method which utilizes a water-soluble substrate (Tween) (23). He also noted some inhibition of liver and kidney staining. In the experiments with the naphthyl substrates, the esterolytic activities of liver and kidney were slightly inhibited, whereas pancreatic activity was markedly accelerated (Table III).<sup>6</sup> This effect is most pronounced with the lauric acid ester. Therefore, an esterolytic enzyme so activated in pan-

<sup>6</sup> The pure stearate, on the other hand, was poorly hydrolyzed by pancreatic tissue and taurocholate stimulation of enzymatic hydrolysis was not observed. However, enzymatic hydrolysis and taurocholate accentuation of hydrolysis were observed with pure  $\beta$ -naphthyl palmitate, m.p. 70–72°, prepared from Eastman palmitic acid, m.p. 61–62°, and by  $\beta$ -naphthyl oleate, liquid, prepared from U. S. P. oleic acid as described above for the laurate.

creas presumably is not present in liver or kidney and is not esterase, but lipase.

*Quinine Hydrochloride*—Rona and Pavlovic (17) discovered that quinine ( $10^{-2}$  M) inhibited the splitting of tributyrin by pancreas but not by liver or kidney. In the experiments given in Table IV, quinine in  $5 \times 10^{-2}$  M concentration inhibited pancreatic hydrolysis of  $\beta$ -naphthyl acetate to a

TABLE III

*Effect of Sodium Taurocholate ( $10^{-2}$  M) upon Esterolytic Activities of Kidney, Liver, and Pancreatic Extracts with Three Substrates\**

Species	Tissue	Experiment No.	$\beta$ -Naphthyl acetate	$\beta$ -Naphthyl laurate	$\beta$ -Naphthyl stearate-palmitate
Rat	Kidney	1	0	0	—
		2	0	0	0
		3	0	—	—
	Liver	1	—	0	0
		2	0	0	—
		3	—	0	0
	Pancreas	1	+	+++	++
		2	+	+++	++
		3	++	+++	+++
Man	Kidney	1	0	0	0
		2	0	0	0
		3	0	—	0
		4	0	—	0
	Liver	1	—	—	—
		2	—	0	0
		3	—	—	—
		4	—	—	—
	Pancreas	1	++	+++	++
		2	+	+++	+
		3	++	+++	0
		4	+	+++	+

\* These experiments were performed as described in Table II with the exception that the color of the ethyl acetate extracts was compared grossly and not colorimetrically. The symbols have the following designation: — = slight inhibition; 0 = no effect; + = slight acceleration; ++ = strong acceleration; +++ = marked acceleration.

moderate degree and of the laurate and stearate-palmitate esters almost completely. But in contrast to their findings (17), quinine also inhibited esterolysis by liver and kidney in varying degree except for the hydrolysis of the stearate-palmitate ester by dog liver and kidney, which was accelerated. This is in agreement with the findings of Rona and Haas (24) that the inhibitory effect of quinine is more pronounced upon the esterolytic action of pancreas (lipase) than of kidney or liver (esterase).

*Sodium Arsenilate*—Rona and Pavlovic (18) noted that atoxyl ( $2 \times 10^{-4}$  M) has a much greater inhibiting effect upon the tributyrinase activity of liver and kidney than upon that of pancreas. The data in Table V with  $10^{-1}$  M concentration of arsenilate support their observations. The inhibitory action of atoxyl on the splitting of short chain carboxylic acid esters also holds for the laurate ester, but somewhat less for the stearate-palmitate ester. On the other hand, although the esterolysis of naphthyl acetate by pancreas is suppressed, that for naphthyl laurate and stearate-palmitate is not. In fact, this cleavage is frequently accelerated. Once

TABLE IV

*Effect of Quinine Hydrochloride ( $5 \times 10^{-2}$  M) upon Esterolytic Activities of Kidney, Liver, and Pancreatic Extracts with Three Substrates\**

Species	Tissue	Inhibition of hydrolysis†		
		$\beta$ -Naphthyl acetate	$\beta$ -Naphthyl laurate	$\beta$ -Naphthyl stearate-palmitate
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Rat	Kidney	27	53	53
	Liver	60 (4)	65	41
	Pancreas	69	93	100 (4)
Dog	Kidney	75	56	0‡
	Liver	85	44 (4)	0‡
	Pancreas	35 (7)	97	95 (4)
Man	Kidney	78	90	81
	Liver	77	94 (4)	85
	Pancreas	30 (4)	90 (4)	89

\* Each number is the average of three experiments except as indicated by the number in parentheses.

† The esterolysis of each substrate by tissue was taken to be 100 per cent and the results in the presence of quinine are expressed as a ratio (per cent) in each experiment.

‡ Indicates acceleration of activity that was visible grossly.

again, marked differences are apparent between the hydrolytic properties of liver and kidney and that of pancreas.

*Sodium Fluoride*—One of the earliest enzyme inhibitors to be studied was sodium fluoride. Hanriot (25) found no effect upon the monobutyrylase of serum, whereas Arthus (26) observed inhibition. Loevenhart and Peirce (27) reported suppression of the activity of hepatic and pancreatic extracts upon ethyl butyrate and olive oil, the effect being 100 to 1000 times greater upon the butyrate ester than on the oil. Rona and Haas (24) reported little influence on the pancreatic enzyme but a marked inhibition of the liver and kidney enzyme. Our results (with 3 mg. per cc. of sodium fluoride) are in essential agreement (Table VI) except in the

TABLE V

*Effect of Sodium Arsenilate ( $10^{-1} M$ ) upon Esterolytic Activities of Kidney, Liver, and Pancreas Extracts with Three Substrates\**

Species	Tissue	Inhibition of hydrolysis†		
		$\beta$ -Naphthyl acetate	$\beta$ -Naphthyl laurate	$\beta$ -Naphthyl stearate-palmitate
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Rat	Kidney	99	98	78 (5)
	Liver	99	100	85
	Pancreas	85	24	0†(4)
Dog	Kidney	95	75	36 (4)
	Liver	94 (6)	85	12
	Pancreas	47 (5)	0†	0†
Man	Kidney	88	92	48
	Liver	96 (5)	78 (4)	82
	Pancreas	27 (7)	0†(4)	0†

\* Each number is the average of three experiments except as noted in parentheses.

† The esterolysis of each substrate by tissue was taken to be 100 per cent and the results in the presence of arsenilate are expressed as a ratio (per cent) in each experiment.

‡ Indicates acceleration of activity that was visible grossly.

TABLE VI

*Effect of Sodium Fluoride (per Cc.) upon Esterolytic Activities of Kidney, Liver, and Pancreatic Extracts with Three Substrates\**

Species	Tissue	Inhibition of hydrolysis†		
		$\beta$ -Naphthyl acetate	$\beta$ -Naphthyl laurate	$\beta$ -Naphthyl stearate-palmitate
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Rat	Kidney	96	90	73 (4)
	Liver	93	76	80
	Pancreas	78	20	37 (6)
Dog	Kidney	92	95	40 (4)
	Liver	88 (4)	80	21 (4)
	Pancreas	5	26 (4)	25 (4)
Man	Kidney	74	75	65
	Liver	81 (4)	79 (4)	87
	Pancreas	10†(7)	0†	0†

\* Experiments performed as described in Table II.

† The esterolysis of each substrate by tissue was taken to be 100 per cent and the results in the presence of fluoride are expressed as a ratio (per cent) in each experiment.

‡ Indicates acceleration of activity that was visible grossly.



case of  $\beta$ -naphthyl acetate in the rat. With this inhibitor, as with sodium taurocholate, quinine, and atoxyl, there is evidence that the pancreas contains one esterase which is similar in its activity upon lower fatty acid esters to the esterase of liver and kidney and which is inhibited by the same agents, while another enzyme, lipase, specific for the pancreas, is more active in hydrolyzing the higher fatty acid esters and is inhibited or accelerated by these agents independently of their effect on esterase.

#### DISCUSSION

Some investigators (28, 29) still use the terms esterase and lipase interchangeably. Three methods recommended for determination of serum lipase utilize the substrates tributyrin (7), olive oil (30), and sorbitan esters of long chain fatty acids (31). A histochemical technique intended to demonstrate lipase by utilizing a polyglycol ester of stearic acid (32) demonstrates an enzyme in the same tissues known to contain esterase by the fact that such tissues also hydrolyze *p*-nitrophenyl propionate (33) and naphthyl acetate (1). Whether the enzymes which hydrolyze the various carboxylic esters are identical or different has not been answered conclusively until now. We believe that there is sufficient evidence to show that the enzymes are not identical and should not be referred to interchangeably.

In the broadest sense, the term esterase refers to any enzyme which can hydrolyze a carboxylic ester linkage. Three classes are referred to in the literature, depending upon the efficiency with which various substrates are hydrolyzed, their distribution in tissues, and the effect of inhibitors upon their activity. They are cholinesterase, non-specific esterase, and lipase. The first obtains its identity because of its capacity to hydrolyze esters of choline. This specificity is not absolute. One of this group shows marked specificity for acetyl choline. Its distribution in tissues as determined by both assay (34) and histochemical methods (35) is quite different from that of non-specific esterase and lipase. And finally the sensitivity of acetylcholinesterase to minute amounts of eserine ( $10^{-5}$  M) further establishes its identity (5, 20).

Theoretically, esters of short chain fatty acids ( $C_2$ - $C_4$ ), such as the *p*-nitrophenyl esters (33) and  $\beta$ -naphthyl acetate, should be acted upon by esterase, while long chain fatty acid esters, such as olive oil and polyglycols of stearic acid, should be hydrolyzed by lipase. But the tissue distribution of lipase with the use of this latter substrate, according to Gomori's histochemical studies (32), is similar to the assay values of Huggins and Moulton (33) and the histochemical visualization of non-specific esterase with naphthyl acetate (1). Although it is possible that two different enzymes have a similar localization in the tissues, there are other conflicting

observations. On the one hand, Cherry and Crandall (30) found that only five tissues were capable of hydrolyzing olive oil significantly, whereas several workers have reported that a fat such as tributyrin is hydrolyzed by many tissues (24, 36, 37). Tributyrin has been used as a substrate for serum lipase (7) and has given values much greater in magnitude (more like those of ethyl butyrate) than those obtained with olive oil as a substrate.

Studies of the relative decomposition rates of various esters by several tissues would be expected to help resolve this problem. Hanriot (25) first pointed out that, as the molecular weight of the fatty acid was increased, hydrolysis by "serum lipase" decreased, while Kastle and Loevenhart (19) noted that with "pancreatic lipase" the reverse was true. Loevenhart (8) showed that the comparable activity upon different esters by the "lipase" of liver, serum, and kidney was quite different from that of the pancreatic enzyme. Two hydrolytic patterns were described by Huggins and Moulton (33), one characteristic of lipase and showing increasing hydrolytic activity with increase of the acyl chain from 2 to 5 carbons, the other being attributable to non-specific esterase in which the 3-carbon acyl chain was hydrolyzed more effectively than the other esters. The work of Balls and Matlack (11, 14) went a step further by comparing the hydrolysis of butyrate and stearate esters. Horse liver, which is rich in ethylbutyrase and tributyrinase, cannot hydrolyze benzyl stearate, while pancreas acts upon all three efficiently. The data we have presented above, which show striking differences in the hydrolysis of  $C_2$ ,  $C_{12}$ , and  $C_{16}$ - $C_{18}$  fatty acid esters of  $\beta$ -naphthol by liver and kidney compared to pancreas in six species, plus the differing effects of inhibitors and accelerators, provide additional proof for the existence of two distinct carboxylic esterases.

At the same time, the data show that there is considerable overlapping in the hydrolytic properties of these enzymes for nearly all carboxylic esters. Accordingly, the concept, implicit in the term "lipase," that this enzyme specifically hydrolyzes fats should be modified. Hydrolysis of simple esters of palmitic and stearic acids is readily accomplished by this enzyme. Apparently the glycerol component plays no determinative rôle. On the other hand, the enzyme which is most active upon fats such as triacetin, tripropionin, and tributyrin is not lipase, but esterase. The most important differentiating factor seems to be the length of the fatty acid. Esterase acts most efficiently upon esters of short chain fatty acids and lipase upon those of long chain fatty acids. However, if these enzymes were mutually exclusive in function, Gomori's histochemical technique with polyglycol esters of stearic and palmitic acids (Tween) should be more specific for lipase than it apparently is. The fact that his substrate is decomposed so satisfactorily by tissues which effect little hydrolysis of

benzyl stearate, monostearin, and  $\beta$ -naphthyl stearate suggests (1) that much of the hydrolysis of Tween is produced by non-specific esterase and (2) that the relative solubilities of this substrate in the aqueous and fat phases may be of importance in determining susceptibility to enzymatic hydrolysis. An ester of a long chain fatty acid made water-soluble because of the presence of numerous hydroxyl groups may bring the substrate into a physicochemical state more favorable for the activity of non-specific esterase. It appears then that simple esters of fatty acids with 8 or more carbons provide factors optimal for lipase cleavage (10).

From our data, it appeared that convenient and specific colorimetric methods for measuring the activity of serum esterase and serum lipase could be developed by the use of the three substrates prepared from  $\beta$ -naphthol. Work based on these considerations is in press (38).

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#### SUMMARY

The esterolytic powers of kidney, liver, and pancreas of six species were studied with three chromogenic substrates prepared from  $\beta$ -naphthol and fatty acids ( $C_2$ ,  $C_{12}$ ,  $C_{16}$ - $C_{18}$ ). Kidney and liver (esterase) hydrolyzed the acetate and laurate. Pancreas (esterase and lipase) hydrolyzed all three substrates. At the concentrations used, eserine inhibited esterase and lipase, arsenilate and fluoride inhibited esterase, quinine inhibited lipase, and taurocholate accelerated lipase and slightly inhibited esterase activity. Evidence is provided for the existence of two distinct enzymes, esterase and lipase. Ideal substrates for the former contain short chain fatty acids, and for the latter long chain fatty acids.

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# METABOLISM OF RABBIT BONE MARROW IN VITRO IN RINGER-BICARBONATE MEDIUM CONTAINING NO ADDED GLUCOSE

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Many cells continue to respire *in vitro* in the absence of exogenous glucose (1). On the other hand, in the presence of added glucose, some tissues show high respiratory rates without carbohydrate utilization (2, 3). In both cases the question of what materials the tissues utilize has not been adequately answered.

It was hoped that a quantitative study of the *in vitro* metabolism of bone marrow in a medium containing no glucose might answer the following questions. Is the rate of oxygen consumption dependent on the presence or absence of glucose? In the absence of aerobic glycolysis, is lactic acid involved in the metabolism of bone marrow cells? How much is the respiratory quotient altered when bone marrow cells are denied glucose?

## EXPERIMENTAL

### Methods

The experimental procedures used in this study have been reported previously in detail (4). Bone marrows, obtained from normal rabbits, were prepared as cellular suspensions in a Ringer-bicarbonate solution at room temperature. No glucose was added to the medium in any of the experiments. During sampling, the cell suspension was stirred constantly. Manometric data were obtained by means of the Summerson constant volume differential manometer (5). Each manometer vessel contained 1.0 ml. of marrow suspension to which was added exactly 1.0 ml. of Ringer-bicarbonate solution. The composition of the Ringer's solution was sodium chloride 8.65 gm. per liter, potassium chloride 0.23 gm. per liter, and calcium chloride 0.24 gm. per liter. Sufficient 0.15 M sodium bicarbonate was added to the Ringer's solution to obtain the desired pH. A gas mixture of 95 per cent oxygen and 5 per cent carbon dioxide was used for equilibration. The pH at the beginning of the experimental period was calculated from the

\* Research Fellow of the United States Public Health Service.

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Henderson-Hasselbalch equation with use of values that were obtained by direct measurement on each manometric system. In all experiments this pH was between 7.4 and 7.1. The experimental period was of 2 hours duration. Manometer vessels were shaken at a rate of 120 oscillations per minute in a constant temperature bath at 37°.

The total contents of each manometer vessel were transferred quantitatively to a 25 ml. volumetric flask and the proteins precipitated by acid tungstate. Chemical analyses were performed on the protein-free filtrates of both the control and the experimental vessels. When the amount of any substance was greater in the control than in the experimental vessel, utilization was considered to have occurred. In this report utilization is indicated by a negative sign. Conversely, production of any substance is indicated by a positive sign. Lactic acid was determined by the method of Barker and Summerson (6). Glycogen was determined by the method of Good, Kramer, and Somogyi (7), and fermentable and non-fermentable sugars by the method of Van Slyke and Hawkins (8). Total reducing substances were determined as glucose by the method of Benedict (9) following acid hydrolysis for 2 hours in a boiling water bath. In these determinations the Klett-Summerson photoelectric colorimeter was used. The analytical accuracy for each set of chemical determinations was estimated by recovery of glucose and lactic acid from samples containing known amounts. For determinations of non-protein nitrogen, two methods were used. Results from both were in close agreement. The first of these was a micro-Kjeldahl method in which ammonia was steam-distilled into boric acid and titrated directly with 0.0143 N sulfuric acid (10). The second was the direct nesslerization method of Koch and McMeekin (11). The quantity of  $\alpha$ -amino acids present in the acid tungstate filtrate was estimated from the carbon dioxide evolved following the addition of chloramine-T (12, 13).

In this report, oxygen consumption is given in c.mm. per mg. of cell protein per hour. Total aerobic acid production is in terms of c.mm. of carbon dioxide liberated from bicarbonate per mg. of cell protein per hour. Analyses of the media are expressed in terms of micrograms of substance per mg. of cell protein per hour. The quantity of cell protein was calculated from the total cell nitrogen by use of the factor 6.25. The total nitrogen was determined in duplicate samples by the micro-Kjeldahl method.

### *Results*

The manometric rates of the present series are compared with those previously reported (4). In both, normal rabbit marrow was studied in a Ringer-bicarbonate medium, but in the previous series the medium con-

tained approximately 250 mg. per cent of glucose. Oxygen consumption of marrow in the presence and in the absence of glucose is compared in Fig. 1. The upper histogram of Fig. 1 shows the distribution of 76 determinations obtained in the present study. The lower histogram gives the distribution and rates of oxygen consumption of rabbit bone marrow in thirty-nine determinations in which glucose had been added to the medium. Ex-

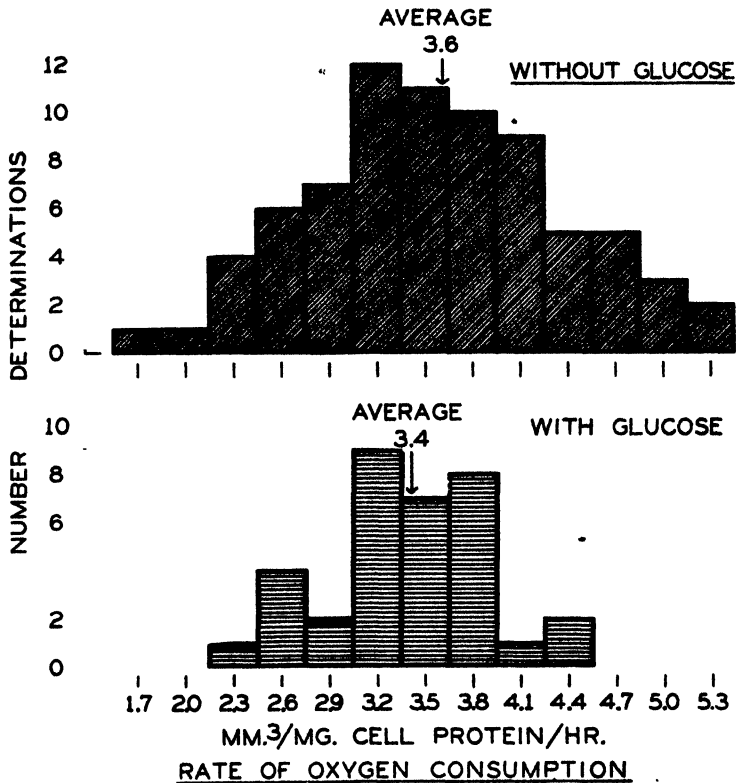


FIG. 1. Comparison of the mean and distribution of rates of oxygen consumption of rabbit bone marrow in the absence and presence of glucose.

cept for the presence of glucose the experimental conditions were the same in the two groups. The distribution of both series is essentially the same. The means average 3.6 and 3.4 c.mm. of oxygen per mg. of cell protein per hour. Hence it appears that bone marrow cells respire as actively in a bicarbonate medium containing no glucose as in one to which glucose has been added.

In the lower histogram of Fig. 2 the respiratory quotient of rabbit bone marrow in a Ringer-bicarbonate medium containing glucose averaged 0.95.



Thirty-nine determinations were made. This value has been interpreted to mean that the tissue utilizes carbohydrate preferentially. When glucose was not added to the medium, the average respiratory quotient fell to 0.84. This is illustrated in the upper histogram of Fig. 2. The fall in respiratory quotient when marrow is deprived of glucose indicates clearly that marrow, like an intact individual, is able to turn to food substances other than carbohydrates.

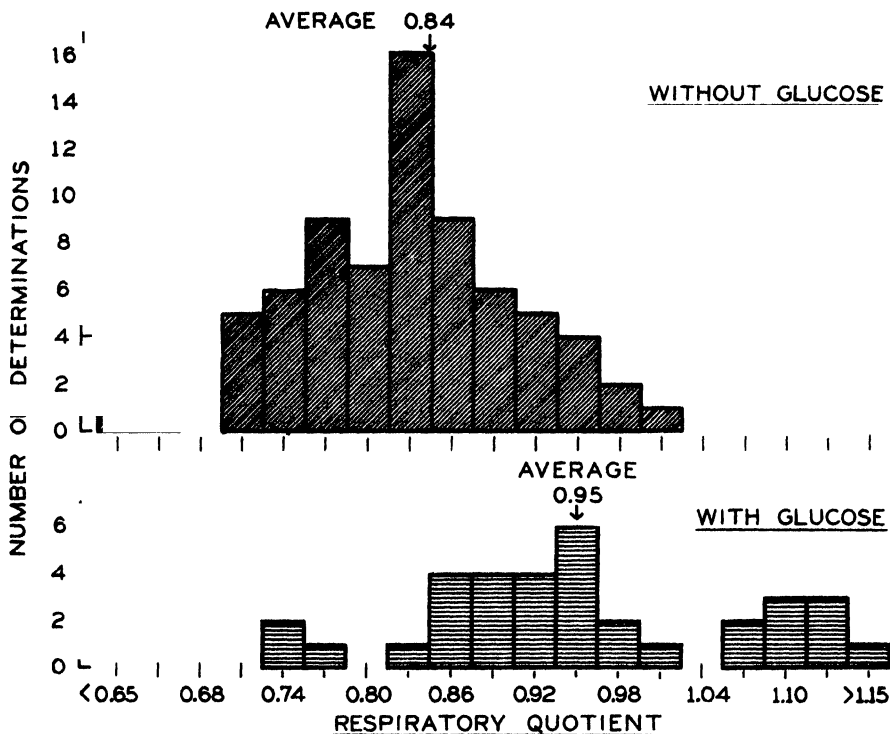


FIG. 2. Comparison of the mean and distribution of the respiratory quotients of rabbit marrow in the absence and presence of added glucose.

Analyses for glycogen and for fermentable and non-fermentable sugars showed that under the conditions of our experiments these substances existed in very small amounts. Therefore it was decided to limit the study of carbohydrate balance to that of total reducing substances and lactic acid. In thirty-nine experiments in which there was no glucose added to the medium, there was an average utilization of 0.9  $\gamma$  of total reducing substances per mg. of cell protein per hour. The recovery error averaged 2 per cent. The distribution and average of these analyses are presented in

Fig. 3 where each column represents the total number of determinations within a 1  $\gamma$  range.

Manometric determinations for total aerobic acid production gave evidence that acid was being formed in small but consistent amounts despite the absence of glucose in the medium. In 76 experiments, this averaged +0.4 c.mm. of carbon dioxide liberated from bicarbonate per mg. of cell protein per hour. This low aerobic acid production directs attention to the results of analyses for lactate.

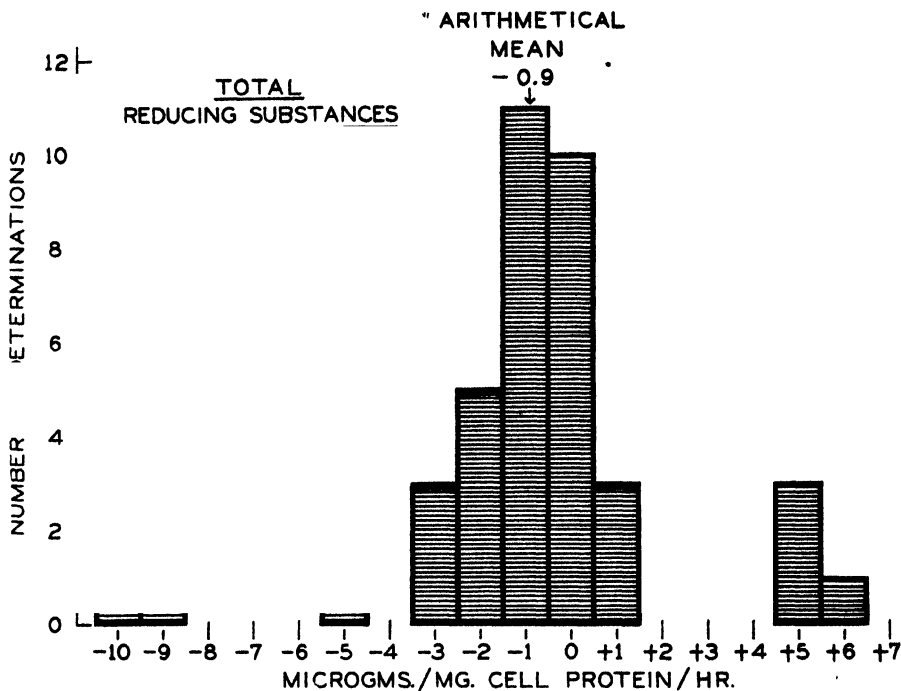


FIG. 3. The distribution and mean of the change in quantity of total reducing substances during a 2 hour experimental period. On the abscissa, a negative sign indicates utilization by marrow; a positive sign, production.

The distribution of values for lactic acid is given in Fig. 4. The average recovery error was 4 per cent. In 67 analyses there was an average utilization of 0.9  $\gamma$  per mg. of cell protein per hour. This quantity was estimated to represent between 15 and 30 per cent of the lactic acid present in the tissue at the beginning of the experimental period. Examination of the individual determinations shows that there was utilization of more than 0.5  $\gamma$  of lactic acid per mg. of cell protein per hour in forty-one cases, and apparent production of more than 0.5  $\gamma$  in eleven. The remaining fifteen determinations fell in the range of -0.5 to +0.5  $\gamma$ .

In an attempt to demonstrate protein metabolism, analyses were made for non-protein nitrogen, total amino acids, and urea. In twenty-nine experiments we were not able to find any consistent indication of production or utilization of non-protein nitrogen. There was apparent production in eleven experiments, apparent utilization in eleven, and in seven there was no measurable difference in the quantities of non-protein nitrogen in experimental and control vessels. Eighteen analyses for amino acids indicated that there was no change in the quantity of these substances in the tissues during the experimental period. A few urea determinations on

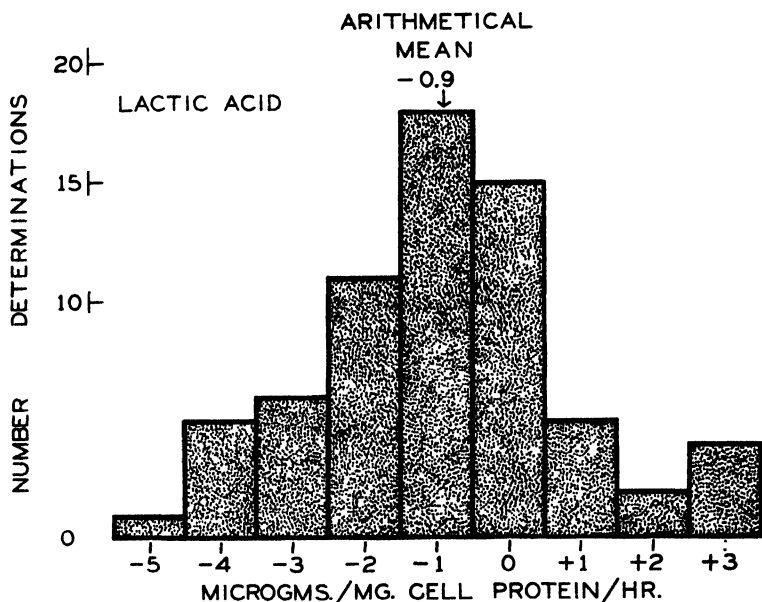


FIG. 4. The distribution and mean of the change in quantity of lactic acid during a 2 hour experimental period. On the abscissa, a negative sign indicates utilization by marrow; a positive sign, production.

the acid-tungstate filtrates showed that this substance was present only in trace amounts. We have interpreted the results of all these analyses to mean that significant protein breakdown does not occur in bone marrow under the conditions of our experiments.

#### DISCUSSION

The rate of oxygen consumption of excised tissue is the usual criterion for determining the level of its metabolic activity. It is clear from our results that the deprivation of glucose does not affect this metabolic capacity of bone marrow. The question is raised as to whether the endog-

enous carbohydrates in marrow are sufficient to maintain the rate of oxygen consumption reported here. The utilization of total reducing substances during the 2 hour experimental period was from 15 to 30 per cent of the total quantity present in the tissue. At this utilization rate, 7 to 14 hours would be required to exhaust completely the endogenous supply of carbohydrates. In four manometric experiments performed under sterile conditions and in which the medium contained no glucose, we have observed that oxygen continues to be consumed at a sustained, linear rate for at least 30 hours. Dickens and Greville (1) have shown that embryonic tissue and Jensen rat sarcoma show little or no fall in respiration when these tissues are deprived of glucose. This is in contrast to brain cortex, retina, testis, liver, kidney, and spleen, which all show a variable reduction of oxygen consumption in the absence of glucose. Furthermore, Summerson, Gilder, and Lee (14) have shown that exogenous glucose does not participate in the oxidative metabolism of mouse lymphosarcoma. It appears, then, that rabbit bone marrow, like many embryonic tissues, is not dependent on a single foodstuff for the maintenance of its metabolic activity.

The utilization of lactic acid under the experimental conditions employed does not make marrow unique. It has been shown that heart muscle (15) consumes lactate even in the presence of abundant glucose. Indeed such may be the case with marrow, for in the presence of exogenous glucose, the lactate utilization may be obscured by active aerobic glycolysis. The total aerobic acid production observed manometrically must be explained by the formation of acids other than lactic acid. Goldinger, Lipton, and Barron (16) have reported the formation of citric acid by rabbit bone marrow.

In the absence of exogenous glucose the respiratory quotient of 0.84 could indicate the oxidation of protein or the oxidation of a combination of carbohydrates, protein, and fats. Our analyses for amino acids, non-protein nitrogen, and urea give no evidence for significant protein breakdown by bone marrow. Combined carbohydrate and lactic acid utilization can account for approximately 38 per cent of the oxygen consumption during a 2 hour experimental period. We believe that the remaining 62 per cent of the oxygen consumption is concerned with the metabolism of fatty substances and suggest that, under our experimental conditions, marrow utilizes carbohydrates and fats in the ratio of 38:62. According to the Zuntz-Schumburg-Lusk table (17) a non-protein respiratory quotient of 0.84 indicates the oxidation of carbohydrates and fats in the ratio of 45:55. The similarity of this ratio to our suggested ratio of 38:62 gives strong support to the idea that fatty substances may be utilized by the marrow. Additional support for the utilization of fat by marrow is to be found in the recent work of Barron (18).

Direct evidence of fat utilization by marrow is now being sought. We

have observed that the oxygen consumption appears to be associated with the fatty portion of bone marrow. When the marrow was suspended in Ringer's solution and centrifuged, three layers were formed. The top layer consisted of fatty material including many marrow cells. The bottom layer consisted principally of packed marrow cells. Manometric observations on the two layers showed that the cellular constituents of the fatty material maintained their normal oxygen consumption, whereas there was virtually no oxygen consumption by the packed cells.

Warren has reported (19) that the metabolic characteristics of marrow vary according to its cellular composition. Predominantly erythroid marrow was characterized as having essentially no aerobic glycolysis, whereas predominately myeloid marrow was shown to possess marked aerobic glycolytic activity. The question arose as to whether marrows with unusual erythroid-myeloid ratios would exhibit the same metabolic pattern in the absence of glucose as that reported above for normal marrow. To answer this question, sixteen experiments were performed but are not reported here in detail. The cellular composition of the marrows varied from 90 per cent erythroid to 70 per cent myeloid. Within the range of cellularity studied, the presence or absence of glucose did not affect the rate of oxygen consumption of any individual marrow. Warren's observations concerning the aerobic glycolytic capabilities of red and of white marrow have been confirmed. Two additional observations were made. There was a lowered respiratory quotient in the absence of glucose regardless of cellular composition, and there was utilization of lactic acid when the medium contained no glucose. From these experiments, it is concluded that the effect of the absence of exogenous glucose is the same regardless of the cellular composition of the individual marrow.

#### SUMMARY

1. The *in vitro* metabolism of rabbit bone marrow in Ringer-bicarbonate media containing no exogenous glucose has been compared with the metabolism of marrow in media to which glucose has been added.

2. The average rate of oxygen consumption by bone marrow was approximately 3.5 c.mm. per mg. of cell protein per hour. This rate was not influenced by the presence or absence of glucose.

3. In the absence of glucose, the average respiratory quotient of marrow fell from 0.95 to 0.84. Utilization of carbohydrates, measured as total reducing substances, was sufficient to account for only 19 per cent of the total oxygen consumption. An additional 19 per cent might be accounted for by lactic acid which was utilized in the absence of exogenous glucose. No evidence was obtained which would indicate significant protein breakdown.

4. The results obtained in these experiments are interpreted to mean that bone marrow utilizes fatty substances in the absence of exogenous glucose.

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# STUDIES ON THE INCORPORATION OF INJECTED CYTOCHROME *c* INTO TISSUE CELLS

## I. INJECTION OF NON-RADIOACTIVE CYTOCHROME *c* INTO RATS PREVIOUSLY GIVEN RADIOIRON\*

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A series of papers has recently appeared (1-13) which does not support the assertion (14) that injected cytochrome *c* may be beneficial in various conditions of hypoxia. The enzymatic concept of the therapeutic use of cytochrome *c* postulates that injected cytochrome enters the cells in order to display its action. Whether such penetration is actually possible, however, has not as yet been proved. Analyses reported by Proger, Dekaneas, and Schmidt (15) to demonstrate enhancement of cytochrome *c* concentration in organs after injection do not constitute such proof for several reasons: (a) Their values for the concentration of cytochrome in normal rat tissues are extremely low, and the values for injected animals are, in several instances, lower than the average values for normal animals found by other workers (16-20);<sup>1</sup> (b) insufficient account seems to be taken of the high individual variation of the natural cytochrome level in the case of the injected animals; and (c) the presence of injected material in extracellular fluid is not excluded at the time the samples are taken. Drabkin (6) employed a technique of partial hepatectomy for internal control, and allowed sufficient time between injection and sampling to exclude interference from extracellular material. In these experiments, no evidence could be found of incorporation of injected cytochrome *c* into liver cells. Similar results on kidney were obtained in our laboratory, with unilateral nephrectomy for internal control. Although these analytical techniques clearly demonstrate that there is no long lasting enhancement of the cytochrome level in organs after injection of the pigment, they fail to inform us of what may happen during the first few hours after injection, when the cytochrome content of the extracellular fluid is considerable.

If it is supposed that cytochrome *c* injected during hypoxia would enter

\* A preliminary report of this paper has appeared (*Federation Proc.*, **8**, 183 (1949)).  
The radioiron used in this investigation was supplied by the Carbide and Carbon Chemicals Corporation, Oak Ridge, Tennessee, on allocation from the Isotopes Division, Atomic Energy Commission.

<sup>1</sup> Beinert, H., unpublished material.



tissue cells and participate in intracellular respiration, then it should reach a state in these cells equivalent to their intrinsic cytochrome *c* and should become thoroughly mixed with this. When hypoxia and, therewith, the supposed "need" were terminated, the cell might eliminate any surplus cytochrome *c*, regardless of its origin. Knowledge could be obtained of such mobilization if either the injected or the intrinsic cytochrome was labeled with isotope and the dilution of isotope was determined.

This paper deals with the injection of commercial cytochrome *c* into rats which had received radioiron. Analyses of some organs of these animals for cytochrome *c* and the radioactivity of this cytochrome are given. Unilateral nephrectomy is proposed as a means of obtaining internal controls for cytochrome determinations in kidneys, and data gained by this technique are reported. All values for cytochrome *c* throughout this paper refer to a molecular weight of 13,000 (16, 18, 21).

#### EXPERIMENTAL

*Supplementation of Rats with Radioactive Iron*—Animals for this study were available from previous work, briefly described elsewhere (22).<sup>2</sup> Weanling rats were made anemic by being fed an iron-deficient diet, together with repeated bleeding. These rats were then injected subcutaneously and intraperitoneally with radioiron for about 12 weeks. The seven male rats used in the present study belonged to two different litters. Data for weight and hemoglobin level at different stages of the experiment are included in Table I. An approximate total dose of radioiron corresponding to  $55 \times 10^5$  counts per minute was injected into each animal. About  $45 \times 10^5$  c.p.m., or 82 per cent, were recovered in hemoglobin and  $0.3 \times 10^5$  c.p.m., or 0.55 per cent, in cytochrome *c*.

*Unilateral Nephrectomy, Cytochrome Injection, and Exposure to Hypoxia*—The right kidneys of two litter mate pairs<sup>3</sup> were removed under ether anesthesia, freed from blood clots and fat, well washed, blotted, weighed, and frozen. The animals were allowed 16 hours to recover from the operation. One animal of each pair was injected, through the tail vein, with cytochrome (Viobin) in saline. The control animal was injected with the corresponding quantity of saline alone (*cf.* Table I). Immediately thereafter, both members of each pair were placed in a low pressure chamber and the pressure was decreased to an equivalent of 20,000 feet in the course of 30 minutes. 24 hours after injection of cytochrome the rats were decapitated and the remaining kidneys, the hearts, and samples of skeletal muscle were removed and treated in the same manner as the right kidneys.

<sup>2</sup> A more detailed description of this work will be published soon.

<sup>3</sup> The pair of Rats O<sub>1</sub> and O<sub>2</sub> was not subjected to unilateral nephrectomy, but to partial hepatectomy. Otherwise, the treatment was the same.

TABLE I

*Concentration and Specific Activity of Cytochrome c in Kidney, Heart, and Muscle*

Male rats, which had previously been injected with radioiron, received injections of cytochrome *c*, and were exposed to hypoxia after removal of the right kidney; the left kidney was removed 40 hours after the right kidney. Rats O<sub>0</sub> and O<sub>1</sub> were not subjected to unilateral nephrectomy, but to partial hepatectomy. Otherwise, their treatment was the same.

Rat No.	Body weight		Hb	Cytochrome <i>c</i> injected	Saline injected	At 20,000 ft. after injection	Organ analyzed	Organ, wet weight	Cytochrome <i>c</i> found	Specific activity of cytochrome <i>c</i> found	Difference of specific activity		Difference of cytochrome <i>c</i> concentration from right control kidney
	gm.	gm. per 100 ml.	mg.	ml.	hrs.			gm.	γ per gm. wet weight	c.p.m. per mg. cytochrome <i>c</i>	From control litter mate	From right control kidney	per cent
O <sub>7</sub>	33 (a)	4.2					R.kidney	0.89	411	1929 ± 23			
	249 (b)	15.8	0	1.5	3		L. "	0.98	389	2000 ± 21			
	280 (c)						Heart	0.75	543	1362 ± 22			
O <sub>8</sub>	35 (a)	4.4					R.kidney	1.01	442	1913 ± 20	-0.8		
	266 (b)		6	1.5	3		L. "	1.08	465	1950 ± 18	-2.5	+1.9	+5.2
	299 (c)	14.9					Heart	0.82	646	1410 ± 19	+3.5		
M <sub>1</sub>	37 (a)	4.0					R.kidney	0.75	395	2150 ± 29			
	225 (b)	15.4	0	2.0	24		L. "	0.67	390	2204 ± 31		+2.5	-1.3
	238 (c)						Heart	0.65	452	1796 ± 28			
M <sub>4</sub>							Skeletal muscle		71	2270 ± 29			
	38 (a)	4.2					R.kidney	0.81	331	2133 ± 30	-0.8		
	251 (b)		8	2.0	24		L. "	0.80	368	2085 ± 29	-5.4	-2.3	+11.2
	292 (c)	15.1					Heart	0.74	468	1681 ± 25	-6.4		
M <sub>6</sub>							Skeletal muscle		57	2356 ± 33	+3.8		
	35 (a)	4.6											
	242 (b)	14.7											
O <sub>0</sub>	284 (c)	14.6											
	31 (a)	4.2											
	231 (b)	14.6	0	2.5	24		Heart		478	1508 ± 21			
O <sub>1</sub>	282 (c)												
	37 (a)	5.1											
	236 (b)		10	2.5	24		"		462	1486 ± 19	-1.5		
P <sub>1</sub> *	267 (c)	15.2											
	310						R.kidney	1.24	392				
P <sub>2</sub> *							L. "	1.20	411				+4.8
							R. "	1.04	372				
	293						L. "	1.04	382				+2.7

Radioiron injected between (a) and (b); experiment at (c).

\* Not injected with radioiron or cytochrome *c*.

*Analysis of Tissues for Cytochrome c and Radioactivity of Isolated Cytochrome*—Tissues of Rats O<sub>6</sub>, O<sub>7</sub>, O<sub>8</sub>, and O<sub>9</sub> were analyzed by the method of Rosenthal and Drabkin (16) and Rats M<sub>1</sub> and M<sub>4</sub> by the procedure proposed by Carruthers (20)<sup>4</sup> for spectrophotometric cytochrome analysis. The final cytochrome solution obtained from Rats O<sub>6</sub> and O<sub>7</sub> was dialyzed 24 hours against 0.5 per cent NaCl solution. 1.5 ml. of an inactive FeCl<sub>3</sub> solution, containing 1 mg. of iron per ml. and 0.5 ml. of concentrated sulfuric acid, were then added to each cytochrome sample. Final digestion, precipitation of iron, and electroplating were carried out in a manner similar to the procedures described by Peacock *et al.* (23), with the exception that ammonium citrate was used as the plating medium. Counts were taken by means of an argon-filled Geiger tube with a 1 mm. beryllium window.

*Determination of Iron in Isolated Cytochrome*—A pooled sample of six kidneys of Rats O<sub>6</sub>, O<sub>8</sub>, and M<sub>0</sub> was analyzed for cytochrome, iron, and for the radioactivity of a fraction of the isolated cytochrome as well as of the liberated iron. The cytochrome isolated by the procedure of Rosenthal and Drabkin (16) was dialyzed against 0.5 per cent NaCl solution for 24 hours. A slight precipitate (Sample A) was centrifuged. Samples were drawn for spectrophotometric analysis (Sample B) of cytochrome and for iron determination. The precipitate (Sample A) was dissolved in 1 N sodium hydroxide, diluted, and its cytochrome content and radioactivity determined. The method of Drabkin (24) was adapted to the determination of iron in cytochrome samples of low purity by including a trichloroacetic acid precipitation of proteins. For example, the mixture of cytochrome solution,<sup>5</sup> 4.0 ml., 0.169 N potassium hydroxide, 1.0 ml., and 30 per cent superoxol, 0.25 ml., was digested overnight, and then heated 10 minutes to 90°. 1 N hydrochloric acid, 0.5 ml., was added and heated 10 minutes to 90°; 100 per cent trichloroacetic acid, 0.3 ml., was added, centrifuged, the volume read, decanted into a 10 ml. graduated cylinder, and the volume again read. 0.5 ml. of 2 N sodium hydroxide and 0.2 ml. of 50 per cent ammonium acetate were added to give a pH of 4.0 to 4.2, and 0.2 ml. of 1 per cent ascorbic acid and 1.0 ml. of 0.1 per cent *o*-phenanthroline were added, the color developed for 1.5 hours, and read at 512 mμ in the Beckman spectrophotometer. The final solution (Sample C), as well as the trichloroacetic acid precipitate (Sample D), was quantitatively collected, digested, and plated as described above. From the cytochrome determination in Samples A and B, the iron determination in Sample C, and radioactivity measurements in Samples A, B, C, and D, the content

<sup>4</sup> The proper type of aluminum oxide was kindly supplied by Dr. C. Carruthers.

<sup>5</sup> The NaCl content of blank and iron standard samples in each run was adjusted after the cytochrome solution used.

of cytochrome and non-cytochrome iron of the Precipitates A and D was calculated.

The procedure described for the determination of iron in samples of impure cytochrome was checked independently by recovery experiments. In the method of Keilin and Hartree (25) for the isolation of cytochrome *c*, admixed proteins are precipitated in two steps with ammonium sulfate. Protein obtained from such precipitates during the isolation of cytochrome from rat muscle was dialyzed against distilled water and lyophilized. A 31 per cent pure cytochrome preparation was then assayed for iron with so much of this protein added as to make a 9 per cent cytochrome and with added standard iron solution. Controls with no additions were also analyzed. Good recovery was found in all cases, as can be seen in Table II.

TABLE II

*Recovery of Added Iron in Determinations of Iron in Impure Cytochrome c Preparations*

Added	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
Iron, $\gamma$ . . . . .	10.0			10.0	4.0		4.0
Rat muscle protein, mg. . . . .		20.0		20.0		20.0	20.0
31% cytochrome <i>c</i> , mg.			8.0		8.0	8.0	8.0
Recovered iron, $\gamma$ . . .	10.0	2.0	10.8	11.9	14.9	12.9	17.1

### Results

Comparative determinations of cytochrome *c* in right and left kidneys of rats are given in Tables I and III. The rats in Table III had both kidneys removed simultaneously and those in Table I with a 40 hour interval, the right kidney being excised first. The difference of the cytochrome concentration in the left and right kidney does not exceed  $\pm 5$  per cent (except in one case), which is considered to be the error of the analytical procedure (16). Raska (26) has found considerable increase of the cytochrome concentration in the remaining hypertrophic kidney of unilaterally nephrectomized dogs, but the shortest interval between the removal of the kidneys in his experiments was 6 days. It is possible that the relatively high difference of 11.2 per cent in one instance (Table I) reflects a similar tendency. The additional stress of exposure to hypoxia and consequent low food intake, which the unilaterally nephrectomized animals underwent in our experiments, might well have suppressed rapid hypertrophy within the first 40 hours. It is evident from the given data that unilateral nephrectomy and analysis of the removed kidney afford a useful means of determining changes in the cytochrome concentration of the remaining kidney which occur during the treatment given to the animal

TABLE III  
*Cytochrome c Concentration in Right and Left Whole Kidneys of Rats Removed Simultaneously*

	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5		Rat 6	
	R.*	L.†	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.
Kidney, wet weight, gm.	0.610	0.702	0.648	0.696	0.657	0.632	0.696	0.813	0.800	0.773	0.611	0.701
Cytochrome c, $\gamma$ per gm. wet weight	264	259	245	248	186	195	270	270	222	233	283	295
Difference of cytochrome concentration, %		-1.9		+1.2		+4.8		0		+5.0		+4.2

\*Right kidney.

†Left kidney.

after removal of the control kidney, provided this interval is kept short. Using this technique, we found (Table IV) that 30 minutes after injection of 4 mg. of cytochrome to a 180 gm. rat 8.9 per cent of the injected dose was in the remaining left kidney, the cytochrome content of this kidney being increased by 305 per cent. In a similar experiment on a 210 gm. rat we found after 2.5 hours 3.6 per cent of the injected dose in the left kidney, corresponding to an increase of 75 per cent of its original cytochrome content. The values for the cytochrome concentration in the hearts of these animals were entirely within the normal range for rat heart. This is in agreement with the findings of Drabkin (6) that the kidney is the only organ analyzed so far where accumulation of cytochrome *c* after injection can be shown with certainty by normal means of analysis. The

TABLE IV

*Concentration of Cytochrome c in Whole Kidneys and Hearts of Rats*

Right kidney removed; rats injected intravenously with 4 mg. of cytochrome immediately after nephrectomy.

Body weight	Kidney, wet weight		Heart, wet weight	Hrs. between removal of right and left kidney	Cytochrome c								Injected dose present in left kidney
	Right	Left			In kidney				In heart	Calculated for left kidney before injection	Enhancement in left kidney		
					Right	Left	Right	Left			γ total	per cent	
gm.	gm.	gm.	gm.		γ per gm. wet weight	γ per gm. wet weight	γ total	γ total	γ per gm. wet weight	γ total	γ total	per cent	per cent
180	0.542	0.598	0.469	0.5	195	792	106	474	395	117	357	305	8.9
210	0.666	0.703	0.615	2.5	274	482	183	339	426	193	146	76	3.6

tremendous increase of the cytochrome content of 305 per cent after 30 minutes, as compared to about 100 per cent reported by Drabkin, is explained by the fact that he used intraperitoneal injection into rats having both kidneys intact, whereas we injected intravenously into unilaterally nephrectomized rats. The difference between these two procedures is even more striking, since we injected only 4 mg. in contrast to 15 mg. injected by Drabkin. Drabkin found 14 per cent of the injected dose in the urine of his rats. We observed, as a rule, a distinctly reddish urine shortly after injection and found, by direct spectrophotometry, 24, 27, and 32 per cent respectively of the injected dose (5 mg.) in the first urine of three rats. A complete absorption curve was taken, which left no doubt as to the identity of the absorbing pigment with cytochrome. It is difficult to understand the failure of Proger and coworkers (15) to detect any cyto-

chrome in the urine of their animals, which were injected with similar or even larger doses.

Table I shows values for the concentration and radioactivity of cytochrome *c* in rats which have been raised under supplementation with radio-iron, nephrectomized unilaterally,<sup>3</sup> injected with commercial cytochrome, and kept at simulated altitude, as described. The litter mate pairs of Rats O<sub>6</sub> and O<sub>7</sub>, M<sub>1</sub> and M<sub>4</sub>, and O<sub>8</sub> and O<sub>9</sub> were treated together. The cytochrome *c* concentration in the remaining kidneys after injection is found to be at the base-line level as given by the previously removed control kidney. This indicates that the 24 hour interval between the injection and death of the animals was sufficient to clear cytochrome from the extracellular fluid. In the case of Rat M<sub>4</sub> there is some doubt in this regard as to the reason for the 11 per cent higher cytochrome found in the remaining kidney. This could be explained as either (a) injected cytochrome *c* left in extracellular spaces, (b) the immigration of injected cytochrome, (c) an actual increase of intracellular cytochrome in close relation to hypertrophy (26, 27), or (d) a great difference in the cytochrome content of left and right kidneys in this rat. In the first two instances, we would certainly expect to find 11 per cent lower specific activity of the cytochrome in the left kidney; in (c) we would expect to find a lower specific activity if the injected pigment is utilized and incorporated during the process of hypertrophy; and in (d) we would expect the same specific activity in the left kidney as in the right control kidney. As a matter of fact, the specific activity is found to be practically identical in both kidneys, which would mean that either the 11 per cent difference in cytochrome content existed before or, as we are more inclined to believe, some hypertrophic enhancement of the cytochrome concentration took place, which, however, made no use of the injected material. Thus, the finding of an unchanged specific activity despite a somewhat increased cytochrome level after injection is the more convincing because no injected cytochrome has been incorporated. It must be emphasized that our method of unilateral nephrectomy does not reveal any hypertrophic process taking place in the remaining kidney. It shows us only that, if the organ is enlarged hypertrophically, this process usually does not change the ratio of cytochrome content to tissue wet weight; nor does it change the specific activity of the cytochrome. Since the animals had not been given radioactive iron for several weeks preceding this experiment, any newly formed cytochrome in the hypertrophic kidney would be expected to be of lower specific activity than the pigment present before. The over-all accuracy of the procedure, by which the values for the specific activity are obtained, is considered to be  $\pm 3$  per cent. The difference of the specific activity of the heart cytochrome of Rats M<sub>1</sub> and M<sub>4</sub> borders on what may be considered significant; but it should be borne in mind that two different animals are compared in the

reported heart and muscle analyses, and less emphasis is placed on these results than on those obtained on the kidneys of one animal. On the other hand, one has difficulty in believing that a 6.5 per cent enhancement of the cytochrome concentration in heart could be of any benefit, inasmuch as the individual variation of the cytochrome content of rat hearts is considerably higher. One would hardly consider Rats M<sub>1</sub>, M<sub>4</sub>, O<sub>6</sub>, and O<sub>7</sub> to be cytochrome-deficient compared with Rats O<sub>8</sub> and O<sub>7</sub>, which have a 15 and 35 per cent higher cytochrome concentration in their hearts.

The close agreement of all values for the specific activity of cytochrome of corresponding organs indicates that the procedures used are reproducible. This is even more conclusively borne out by the fact that, for the pairs of Rats O<sub>6</sub> and O<sub>7</sub> and M<sub>1</sub> and M<sub>4</sub>, two different procedures for the isolation of cytochrome were used, Rosenthal and Drabkin's method (16) involving ammonium sulfate precipitation of contaminating proteins and Carruthers' method (20) applying adsorption of cytochrome on aluminum oxide and decantation of contaminants.

Further proof of the validity of the applied procedure is furnished by the determination of iron in a kidney sample obtained by the analytical method of Rosenthal and Drabkin. For 1 mg. of pure cytochrome in the preparation, as indicated by spectrophotometric analysis,  $4.3 \pm 0.1 \gamma$  of iron were found. This is true for the dialyzed preparation as described above under "Experimental." Cytochrome determinations and radioactivity measurements of the precipitate formed during dialysis and of the trichloroacetic acid precipitate obtained during iron analysis showed that the former contained 0.25  $\gamma$  of iron per mg. of pure cytochrome in the original total sample, which could not be accounted for by the cytochrome, and the latter contained practically no unaccountable iron. Since in the experiments of Table I the total proteins of the final trichloroacetic acid precipitation in the analytical method for determination of cytochrome were ashed and counted, whether dialyzed or not, we must add this 0.25  $\gamma$  to the 4.3  $\gamma$  per mg. of cytochrome for these samples, so that they would contain  $4.55 \pm 0.1 \gamma$  of iron per mg. of cytochrome *c*. This gives about 6 per cent as unaccounted for by cytochrome. However, precipitates formed during dialysis of cytochrome consist, to a considerable extent, of denatured cytochrome, so that 6 per cent may be considered rather as the upper limit for non-cytochrome iron in this fraction. It seems justifiable, therefore, to say that nearly 95 per cent of the measured radioactivity in the cytochrome samples stemmed from cytochrome iron.

#### DISCUSSION

It was found that about 30 per cent of the injected material is excreted in the first urine after injection and that 8.9 and 3.6 per cent respectively



of the injected dose are present in the left intact kidney 0.5 and 2.5 hours after unilateral nephrectomy and injection of 4 mg. of cytochrome. Thus, from 6 mg., which were used as the smallest dose in the crucial experiments, about 2 mg. would be excreted rapidly and about 500 and 200  $\gamma$  respectively would be in the left kidney 0.5 and 2.5 hours after injection. In a kidney of 1 gm., wet weight, and 450  $\gamma$  of cytochrome, this would mean a dilution of over 100 and of 45 per cent respectively at these times of the radioactivity of the intrinsic cytochrome. If only one-tenth of the 500  $\gamma$  present in the kidney after 30 minutes had entered the cells, we should find an 11 per cent dilution of the radioactivity, which would still be well above the limit of error of the analytical methods used. A 5 per cent dilution could not be established with certainty, but it is thought that an incorporation of 5 per cent more cytochrome would be of little value for the organism for reasons given above under "Results."

The present negative results were obtained, despite exposure of the injected animals to hypoxia, immediately after injection, when a need for more respiratory catalysts should have arisen if respiratory catalysts are indeed the limiting factors under such conditions. The simulated altitude of 20,000 feet applied in our experiments is certainly easily tolerated by the rats. We did not dare, however, to expose our animals to a higher altitude so soon after the operation, and it was evident by observation that the chosen altitude was sufficient to place a stress upon these animals. It was observed, furthermore, that the animals receiving no cytochrome were in better shape than their cytochrome-injected litter mates. This is in agreement with results obtained by Christensen and Clinton (4) and by Mangun (9).

Considering the therapeutic value of cytochrome *c* on the basis of the present results, it should be remembered that our approach does not preclude the possibility that injected cytochrome may have some action, perhaps a rather unspecific one, entirely apart from its well established classical function as an electron carrier in cellular oxidation-reduction systems. There seems to be at least some evidence which might point in that direction (6, 28). Baumberger, Leong, and Bardwell (29) have discussed the peculiar action of hemoglobin on the respiration of yeast cells, which seems different from its classical function as an oxygen carrier. Our experiments deal only with the original concept of a utilization of injected cytochrome as a catalyst in cellular respiration.

The difference in the specific activity of hearts and kidneys is remarkable. Iron analysis in kidney samples shows that this difference cannot be due to iron-containing impurities. It rather seems to originate from differences in iron utilization.

## SUMMARY

1. The difference between the cytochrome concentration of the right and the left kidney of a rat does not generally exceed 5 per cent, whether the kidneys are removed simultaneously or with a 40 hour interval. Unilateral nephrectomy is, therefore, a useful means for establishing an internal control for changes in the cytochrome concentration of the remaining kidney.

2. By this technique a 305 per cent increase in the cytochrome level was found 0.5 hour after injection of 4 mg. of cytochrome and a 75 per cent increase after 2.5 hours in kidney. Up to 30 per cent of the injected cytochrome was found in the first urine after injection.

3. Rats which had received radioiron while on an iron-deficient diet were nephrectomized unilaterally, injected with commercial cytochrome *c*, and exposed to a simulated altitude of 20,000 feet for 3 and 24 hours respectively. No significant elevation of the cytochrome concentration in kidneys, hearts, and muscles was found 24 hours after injection and no significant dilution of the specific activity of the isolated cytochrome.

4. A modification of Drabkin's method for determination of iron in cytochrome is proposed, which is applicable to cytochrome samples containing considerable protein impurities. The validity of the procedure is checked by the recovery of added iron and the distribution of radioactivity.

5. The present experiments do not give any evidence of incorporation and utilization of injected cytochrome *c* in tissue cells. The conclusions to be drawn from these results on the therapeutic use of cytochrome injections are discussed and the limitations of such conclusions pointed out.

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# SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF FREE PENTOSE AND PENTOSE IN NUCLEOTIDES\*

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In preceding papers several color reactions of various sugars (desoxypentoses (1), glucuronic acid (2), galacturonic acid (3), methylpentoses (4)) with SH compounds in sulfuric acid were described. All of these reactions, specific for certain classes of sugars, consist of at least two consecutive reactions, namely an initial breakdown of the sugar under the influence of strong acid to a highly reactive product, which combines with the SH compound to form a colored product. The present report deals with a new special form of the cysteine reaction of sugars. In this reaction every class of sugars (desoxypentoses, pentoses, methylpentoses, hexoses, hexuronic acids) yields reaction products of sufficient, though unequal, stability and with a characteristic absorption curve. This makes it possible to detect by spectrophotometric measurements every class of sugars present in single samples of a given solution and determine them quantitatively in most cases. In this report only the determination of small amounts of pentoses in free or combined form in the presence of other sugars is discussed in detail. It appears probable that the reaction products of sugars in modifications of the cysteine reaction previously described either are identical with those of the same sugars in our new reaction or are derived from them by secondary reactions. The new reaction of sugars can therefore be regarded as the basic form of the cysteine reaction of sugars and will be designated by BCyR.

## EXPERIMENTAL

### *Two Steps of BCyR*

*Procedure*—To 1 cc. of a solution containing 10 to 50  $\gamma$  of sugar in a test-tube are added, with cooling in tap water, 4 cc. of concentrated sulfuric acid. The mixture is shaken vigorously and repeatedly, and replaced in a cold water bath. When the reaction mixture is cooled to room tem-

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perature, the decomposition of the sugar ceases, and the reaction product remains unchanged for hours. The reaction mixture is colorless but shows in the ultraviolet a strong absorption which rises abruptly from 350  $m\mu$  to a maximum between 320 and 330  $m\mu$  or below.<sup>1</sup> The maximum differs with different classes of sugars (Fig. 1).

*Reaction of Breakdown Products of Sugars with Cysteine*—When 0.1 cc. of 3 per cent cysteine hydrochloride is added to the cooled reaction mixture, the breakdown products of sugars combine with it to yield compounds with an absorption spectrum different from that of the initial breakdown

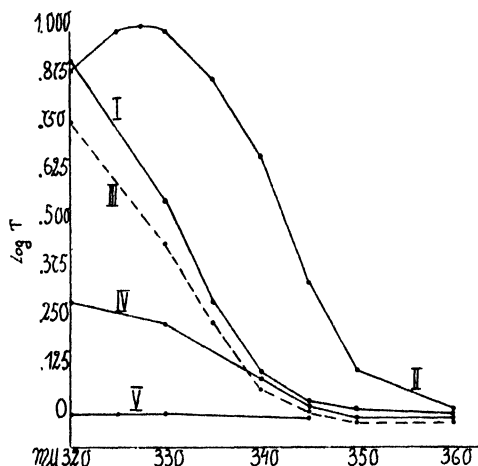


FIG. 1. Absorption curves of primary breakdown products of BCyR of various sugars in  $H_2SO_4$  before addition of cysteine. Curve I, ribose, 5 mg. per cent; Curve II, fucose, 5 mg. per cent; Curve III, furfural, 1 mg. per cent; Curve IV, glucose, 5 mg. per cent; Curve V, desoxyribonucleic acid, 25 mg. per cent.

product and different for every class of sugars. The reaction sets in immediately, but its velocity is different for various sugars. In the case of pentoses, hexoses, and hexuronic acids the reaction with cysteine is practically completed after 10 minutes at room temperature. With methylpentoses many hours are required and with desoxyribose the reaction is completed only after several days. The products of the reaction with cysteine have all their absorption maxima between 375 and 410  $m\mu$ . The progress of this reaction is accompanied by a rapid decrease of the absorption around 320 and 330  $m\mu$ , characteristic for the primary breakdown products of sugars and can thus easily be measured quantitatively.

<sup>1</sup> The ultraviolet absorption spectra of breakdown products of sugars in concentrated  $H_2SO_4$  were first observed and studied by Bandow (5) and under different conditions later by Holzman, MacAllister, and Niemann (6).

*Absorption Spectra of Reaction Products of BCyR of Sugars*—Fig. 2 contains the absorption spectra of the reaction for five types of sugars 10

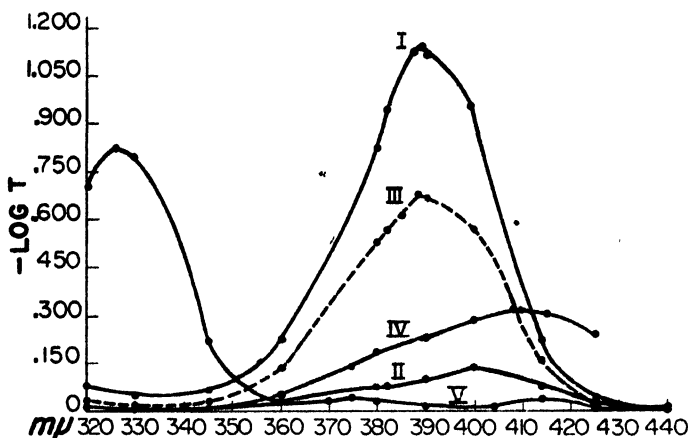


FIG. 2. Absorption curves of reaction products of BCyR of various sugars 10 to 30 minutes after addition of cysteine. Curve I, ribose, 5 mg. per cent; Curve II, fucose, 5 mg. per cent; Curve III, furfural, 1 mg. per cent; Curve IV, glucose, 5 mg. per cent; Curve V, desoxyribonucleic acid, 25 mg. per cent.

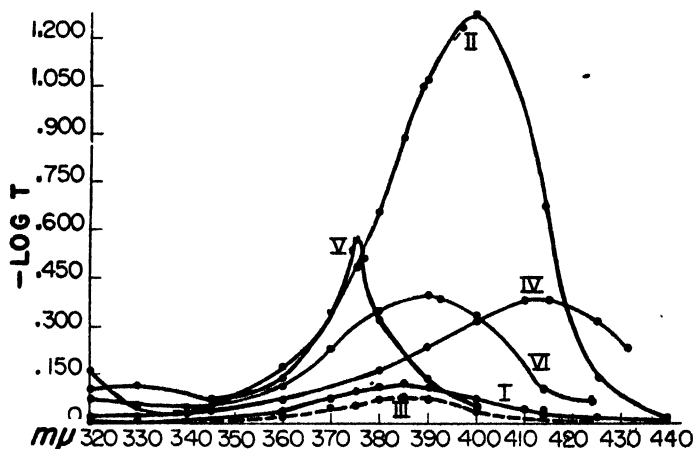


FIG. 3. Absorption curves of reaction products of BCyR of various sugars 24 hours after addition of cysteine. Curve I, ribose, 5 mg. per cent; Curve II, fucose, 5 mg. per cent; Curve III, furfural, 1 mg. per cent; Curve IV, glucose, 5 mg. per cent; Curve V, desoxyribonucleic acid, 25 mg. per cent; Curve VI, xylose, 5 mg. per cent.

to 30 minutes after addition of cysteine, and Fig. 3, 24 hours later. The absorption curves from hexuronic acids and pentoses are nearly identical and the peak of their curves lies at 390 mμ. The curves from desoxyribose,

methylpentoses, and hexoses are completely different, with peaks at 375, 400, and 408  $m\mu$  respectively. Sugars belonging to the same classes show maxima at the same wave-lengths. The shape of the absorption curves is characteristic for every class of sugars and essentially identical for all members investigated of the same class, but the absolute intensity of absorption is different for every sugar.

*Sensitivity of Products of BCyR against Water*—The reaction between the primary breakdown products and cysteine differs for various classes of sugars with respect to its velocity, the absorption curve of its product, and the stability of the products. This stability depends on the ratio of water to sulfuric acid in the reaction mixture. The reaction product from pentoses and hexuronic acids is the most unstable. It decomposes rapidly at the original (1:4) ratio of water to sulfuric acid, and  $D_{390}$  decreases by 50 per cent after 2 hours to reach a minimum after 24 hours at room temperature. This rate of decrease is nearly identical for all pentoses and for adenosine-3-phosphate. That this instability of the reaction product of pentoses is due to the water in the reaction mixture can be easily shown by adding various amounts of concentrated  $H_2SO_4$  to the reaction mixture and measuring the rate of decrease of  $D_{390}$ . When the reaction mixture consists of 8 volumes of  $H_2SO_4$  and 1 volume of  $H_2O$ , the reaction product is practically stable for 24 hours.

The reaction product of hexoses remains stable at the ratio 1:4. However, when enough water is added after the completion of BCyR to make the ratio 1:2.5, this reaction product becomes unstable and its absorption at 408  $m\mu$  decreases, reaching a minimum after about 48 hours at room temperature.

The reaction products of methylpentoses and desoxyribose, which are formed much more slowly, show complete stability at the ratio 1:2.5. In the case of methylpentose it becomes unstable at 1:1.6, and  $D_{400}$  in this case sinks to a minimum after 48 hours at room temperature.

*Influence of Concentration of  $H_2SO_4$  on Velocity of BCyR*—Increase of the amount of concentrated  $H_2SO_4$  in the reaction mixture inhibits not only the decomposition of the product of BCyR but also the second step of BCyR itself. While at the original ratio of 1:4 for  $H_2O:H_2SO_4$  BCyR of pentoses is practically completed in 10 minutes, at 1:8 several hours are required. At this ratio the velocity of BCyR of methylpentoses sinks to zero and the absorption at 330  $m\mu$ , owing to the primary reaction product, remains practically unchanged during 24 hours.

*Factors Controlling Intensity of BCyR.* (a) *Temperature and Time of Heating*—The concentration of the primary and final reaction product depends on the temperature attained during the mixing of  $H_2O$  and  $H_2SO_4$  and the time during which the reaction mixture remains at the tempera-

ture necessary for the decomposition of the sugar. The highest values of absorption at  $320\text{ m}\mu$  are, therefore, obtained, when  $\text{H}_2\text{O}$  and  $\text{H}_2\text{SO}_4$  are mixed without any cooling and allowed to cool at room temperature. Cooling with ice gives lower values than with water at  $20^\circ$ . The latter procedure was chosen as most practical. Once the primary breakdown product was formed, it remained unchanged at room temperature. It proved advantageous to wait 2 hours before adding cysteine with frequent shaking to avoid excessive formation of air bubbles.

(b) *Influence of Impurities in Sulfuric Acid on Primary Breakdown of Sugars in  $\text{H}_2\text{SO}_4$* —Certain impurities in concentrated  $\text{H}_2\text{SO}_4$  increase the breakdown of sugars under the influence of acid. This increase is much more marked with hexoses than with other sugars. It is, therefore, advisable to use very pure sulfuric acid (low in N and As).

*Detection of Pentoses, Methylpentoses, and Hexoses in Mixtures with Each Other*

*Principle*—Although all classes of sugars differ from each other by the position of their absorption maxima in BCyR, it is not possible to use these differences for the detection of sugars in mixtures of several classes of sugars. For this purpose two procedures can be applied. (a) The absorption in BCyR is determined at two or more wave-lengths chosen in such a way that the differences of densities at these wave-lengths are positive for one class and zero or negative for all other classes of sugars in solution. (b) Differences in the sensitivity of the velocity of BCyR of various sugars and of the stability of their reaction products are used for their detection.

When desoxypentoses and hexuronic acids have been excluded by the diphenylamine (7) or cysteine reaction (1) and the carbazole reaction (8), the three other classes of sugars can be detected in one single sample in the following way.

*Test for Hexoses*— $D_{440}$  measured 10 minutes after addition of cysteine is practically zero for pentoses and methylpentoses and high for hexoses. In case of doubt the ratio  $D_{440}:D_{330}$  can be used. It is greater than 1 for hexoses and zero or nearly zero for the other two classes of sugars (Table I).

*Test for Pentoses*— $D_{330} - D_{414}$  is negative for hexoses and zero for methylpentoses. It is highly positive for pentoses. Pentoses in presence of 5-fold excess of hexoses and great excess of methylpentoses can be thus detected. If the excess of hexoses is still greater, we use  $D_{330} - D_{414} + D_{440}$ . This expression is only slightly negative for all hexoses, and becomes positive if a pentose in an amount of only one-tenth of that of hexoses is present in solution (Table II).



*Test for Methylpentoses*—After the reaction mixture has been tested for pentoses and hexoses, 0.6 cc. of water is added, with cooling.  $D_{400} - D_{380}$  is

TABLE I

*Density Increments for Various Sugars between 380 and 414  $m\mu$ ,  $\Delta D_{414,380}$ , and Density at 440  $m\mu$ ,  $D_{440}$ , in BCyR 10 to 30 Minutes after Addition of Cysteine*

Experiment No.	Substance	Concentration	$\Delta D_{414,380} \times 1000$	$D_{440} \times 1000$
		mg. per cent		
I	Ribose	5	+958	+7
	Xylose	2.5	+942	+5
	Yeast adenylic acid	10	+640	0
II	Glucose	5	-156	+140
	Galactose	5	-137	
	Mannose	5	-122	+110
III	Fucose	5	-4	+10
	Ribose	5	+660	+3
IV	Glucose	5	-156	
	Fructose	2.5	-251	
	Desoxyribonucleic acid	25	+24	
V	Muscle adenylic acid	20		+17

TABLE II

*Density Increments for Various Sugars between 380 and 400  $m\mu$  10 Minutes and 2 Hours after Addition of 0.6 Cc. of  $H_2O$  to Reaction Mixture of BCyR*

Experiment No.	Substance	Concentration	$\Delta D_{400,380} \times 1000$	
			10 min.	2 hrs.
		mg. per cent		
	Yeast adenylic acid	10	18	8
	Ribose	5	18	6
	Xylose	2.5	20	-3
II	Ribose	5	-4	-5
	Fructose	2.5	177	179
	Desoxyribonucleic acid	25	-21	42
III	Fucose	5	135	424
	Ribose	5	15	11
IV	Glucose	5	96	93
	Mannose	5	108	105
	Galactose	5	85	81

measured 10 minutes and 2 hours after addition of water. This density increment is and remains zero for pentoses and is positive for hexoses and methylpentoses after 10 minutes. However, it decreases slightly for the first two in 2 hours, and increases strongly for the last one. 1 part of

methylpentose can be detected in the presence of a 20-fold excess of other sugars.

### *Quantitative Determination of Pentoses<sup>2</sup>*

For the determination of desoxypentose, methylpentose, hexose, and hexuronic acids the modifications of the cysteine reaction described previously (1, 4, 8) are generally more suitable. BCyR can be used for the determination of pentoses, pentose nucleotides, and nucleic acids in the presence of other sugars.

*Determination of Pentoses in Free Form and Pentose Nucleotides in Mixtures with Hexoses*—When desoxypentoses, methylpentoses, and hexuronic acids are not present in a solution in significant amounts, pentoses can be determined by the density increment between 425 and 390  $m\mu$   $\Delta D_{390,425}$ , which is practically zero for all four hexoses investigated 10 to 30 minutes after addition of cysteine and is highly positive for pentoses and pentose nucleotides. As can be seen from Table III,  $\Delta D_{390,425}$  is proportional to the concentration of pentose in the range of 1 to 10 mg. per cent, and is not significantly influenced by the presence of hexoses. However, various pentoses show different values of  $\Delta D_{390,425}$ . These differences are small between ribose, yeast adenylic acid, and arabinose. For xylose, however,  $\Delta D_{390,425}$  is twice as great as for ribose. On the other hand the value for adenosine-5-phosphate is one-sixteenth of that for adenosine-3-phosphate. It is, therefore, not possible to determine the total amount of pentoses in solutions containing more than one of these sugars by BCyR alone. This reaction can nevertheless be used with advantage in combination with other reactions for the determination of pentoses in mixtures.

The values of  $\Delta D_{390,425}$  for hexoses vary slightly around zero from one experiment to another. These variations are in the same sense for every hexose. However, in any determination it is always possible to find between 420 and 426  $m\mu$  the wave-length at which  $D$  for hexose is equal to  $D_{390}$ . To assure the highest accuracy it is, therefore, advisable to run with every determination two standards, one of a hexose and the other of a pentose, and to find the right wave-lengths around 425  $m\mu$ .

*Determination of Ribose Nucleic Acids in Mixtures with Desoxyribose Nucleic Acids and Hexoses*— $\Delta D_{390,425}$  of ribose nucleic acid (RNA) is one-half that of an equivalent amount of yeast adenylic acid. For desoxyribose nucleic acid (DNA) the value 10 to 30 minutes after addition of cysteine is less than 2 per cent of the value for the equivalent of ribose

<sup>2</sup> Material of biological origin often contains substances which react with  $H_2SO_4$  to form compounds absorbing light around 400  $m\mu$ .  $D_{290} - D_{415}$  due to these reactions with  $H_2SO_4$  alone must be determined before the addition of cysteine and subtracted from  $D_{290} - D_{415}$  determined after the addition of cysteine.

TABLE III

*Densities at 390 and 425 m $\mu$  and Their Differences for Various Sugars in BCyR Readings 15 to 30 Minutes after Addition of Cysteine*

Experiment No.	Substance	Concentration <i>mg. per cent</i>	$D_{390}$ $\times 1000$	$D_{425}$ $\times 1000$	$D_{390} - D_{425}$ $\times 1000$
I	Yeast adenylic acid	5	520	209	311
	Glucose	5	230	232	-2
II	Yeast adenylic acid	8	621	30	591
	" " "	4	312	20	292
	" " "	2	159	13	146
III	Fucose	2.5	80	17	63
	Galactose	5	247	257	-10
	Galacturonic acid	5	99	7	92
	Glucuronic acid	5	14	-3	17
IV	Fucose	5	186	139	47
	Yeast adenylic acid	10	1111	49	1062
V	Galactose	5	212	221	-9
VI	Desoxyribonucleic acid	20	17	6	11
VII	Yeast adenylic acid	10	648	27	621
	Ribose	5	1082	42	1040
	Xylose	5	1860	81	1779
VIII	Yeast adenylic acid	5	381	17	364
	" " "	2.5	192	11	181
IX	Fucose	2.5	28	8	20
	Rhamnose	2.5	35	11	24
	Galactose	5	196	199	-3
	" + fucose	2.5	222	200	22
X	Yeast adenylic acid	10	780	40	740
	" " "	5	370	14	356
XI	" " " (a)	5	350	13	337
	Glucose (b)	5	221	205	+16
	(a) + (b)		584	230	354
XII	Yeast adenylic acid (a)	5	360	132	228
	Mannose (b)	5	281	291	-10
	(a) + (b)		684	456	228
XIII	Muscle adenylic acid	14	125	33	92
	Yeast adenylic acid	7	542	23	519
	Harden-Young ester	22	686	870	-184
XIV	Yeast adenylic acid	5	600	-7	593
	Muscle adenylic acid	10	70	-10	60
	Desoxyribonucleic acid	20	32	16	16
XV	Yeast nucleic acid (a)	10	478	16	462
	Desoxyribonucleic acid (b)	20	23	5	18
	(a) + (b)		528	30	498
	Yeast nucleic acid	5	246	19	227
XVI	" " "	5	226	11	215
	Muscle adenylic acid	10	63	5	58
	Yeast adenylic acid	5	470	36	434

TABLE III—*Concluded*

Experiment No.	Substance	Concentration	$D_{390}$ × 1000	$D_{425}$ × 1000	$D_{390} - D_{425}$ × 1000
		mg. per cent			
XVII	Desoxyribonucleic acid (a)	20	24	6	18
	Ribonucleic acid (b)	8	364	24	340
	Glucose (c)	4	234	232	2
	(a) + (b)		397	30	367
	(a) + (b) + (c)		652	265	387
	Desoxyribonucleic acid	40	48	12	36

nucleic acid and is proportional to its amount. This is due to the fact that the reaction product of desoxyribose nucleic acid in BCyR shows an absorption maximum at 375  $m\mu$  and some absorption at 390  $m\mu$ . As DNA can be determined by its diphenylamine or cysteine reaction, RNA can be easily determined in the presence of large excess of hexoses and DNA. To this end BCyR is carried out on the unknown and on three standard solutions of DNA, RNA, and glucose of appropriate concentration. The wave-length at which  $D$  for glucose is equal to  $D_{390}$  ( $D_{425}$ ) is found.  $D_{390} - D_{425}$  is determined for the unknown, the RNA and DNA standards. From the first value the value of  $D_{390} - D_{425}$  due to DNA in the unknown is deducted. The ratio of this difference to  $D_{390} - D_{425}$  of the RNA standard gives the concentration of the latter in the unknown.

#### *Determination and Detection of Sugars in Polysaccharides*

Hexoses and methylpentoses in free form in solution can also be determined quantitatively by the optical density at 408 and 400  $m\mu$ . In mixtures these sugars show an additive behavior. To determine whether sugars bound in polysaccharides can be detected and estimated without prior hydrolysis by BCyR, solutions of the following five polysaccharides were compared with solutions of equivalent amounts of respective free sugars: (1) amylose; (2) bacterial dextrosan freed from levulose; (3) liver glycogen (about 99 per cent pure); (4) blood group substance A; and (5) blood group substance O.<sup>3</sup>

The last two polysaccharides were prepared from hog stomach mucosa. Their content in fucose and galactose was determined by the corresponding cysteine reactions described in preceding papers.

Preparations 1 to 3 showed exactly the same  $D_{400}$  as did an equivalent amount of glucose. Preparations 4 and 5, however, show a much lower absorption at 400  $m\mu$  than was calculated from their content in fucose and

<sup>3</sup> I am greatly indebted to Dr. S. Hestrin for the preparations of amylose and dextran and to Dr. E. Kabat for the preparations of blood group substances.

galactose. The velocity of the reaction of the primary product with cysteine was measured for these two preparations and compared with that of a corresponding sugar mixture. This velocity was found lower in both polysaccharides than in corresponding sugar mixtures. But while the percentage decrease in the concentration of the final reaction product of CyR, as compared with free sugars, was about the same in the two immunologically different polysaccharides, they differed considerably in the velocity of CyR. It is, therefore, possible to detect fucose in those two polysaccharides but not to determine its absolute amount with the help of BCyR. In this respect BCyR differs fundamentally from all other cysteine reactions described.

#### DISCUSSION

The original view that the color reactions of sugars with certain aromatic compounds in acids are due to the formation of furfural or its homologues and their subsequent combination with the aromatic developer proved to be inadequate as a general explanation of this type of reaction. Thus adenosine-5-phosphate was shown to give, under certain conditions, a much stronger orcinol reaction than other pentoses and nucleotides (9), though it yields much less furfural when heated with acid. In the reaction with carbazole (10) and sulfuric acid mannose yields a product with an absorption spectrum completely different from that obtained with glucose. Hexuronic acids in the same reaction (8), under certain conditions, yield a product completely different from that of pentoses, though like pentoses they produce furfural when heated with acids. These observations led to the conclusion (8) that the action of strong acids on sugars produces, apart from furfural and its homologues, a large number of intermediary products related to or derived from furfural. The nature and the relative amount of these products depend on the structure of the sugar. Stacey *et al.* (11) in fact have shown that in the diphenylamine reaction of desoxyribose ten different reaction products are formed, of which only one combines with diphenylamine.

Our observations on BCyR and other forms of the cysteine reaction of sugars support this concept of the mechanism of these types of reactions. In BCyR, the primary breakdown of the sugar at high temperature is dissociated from the following reaction of this product with cysteine, which goes on at room temperature, and can be followed spectrophotometrically. The absorption curves of the final reaction product of members of the same class of sugars are of identical shape. The same is true for the speed of the destruction of this product under the influence of water. This suggests that the primary breakdown product is the same for all members of a class of sugars, and may be identical with furfural in the case of pentoses

and hexuronic acid, and with homologues of furfural in the case of other sugars. The fact that adenosine-5-phosphate yields only about one-sixteenth as much of the primary breakdown product as all other pentoses and adenosine-3-phosphate and that fructose yields 3 times more of it than glucose is in agreement with this assumption, because these yields correspond to the yields of furfural and hydroxymethylfurfural under the influence of strong acids in these cases. This view is further strongly supported by experiments on pure furfural, freshly distilled *in vacuo*. To avoid as far as possible any change of furfural by  $\text{H}_2\text{SO}_4$  at high temperature, 0.1 cc. of 0.01 per cent solution of furfural was added to a mixture of 0.9 cc. of  $\text{H}_2\text{O}$  + 4 cc. of  $\text{H}_2\text{SO}_4$  previously cooled to  $0^\circ$ . This mixture showed essentially the same absorption curve between 320 and 350  $\text{m}\mu$  as the mixture of 1 cc. of a 0.005 per cent ribose solution and 4 cc. of  $\text{H}_2\text{SO}_4$  added with cooling in water at  $22^\circ$  (Fig. 1). After addition of cysteine the absorption in the ultra-violet region of both mixtures decreased at practically the same speed and a new product with the identical absorption maximum at 390  $\text{m}\mu$  developed (Fig. 2). When 0.6 cc. of  $\text{H}_2\text{O}$  was added to both reaction mixtures with cooling in tap water, the reaction product with cysteine disappeared in both cases at the same rate and  $\Delta D_{390, 425}$  became zero at practically the same time. A new red compound appeared instead in both mixtures. These facts leave little doubt that the characteristic primary breakdown product of pentoses in BCyR is mainly furfural, which combines with the added cysteine to form a new compound with the absorption maximum at 390  $\text{m}\mu$ .

The similarity in the shape of the absorption curve of the primary breakdown product of methylpentoses and hexoses as well as that of their reaction product with cysteine and the sensitivity against water of the latter suggest that these primary breakdown products are methyl- and hydroxymethylfurfural.

The varying sensitivity to water of reaction products of BCyR may partially explain the specificity and the mechanism of those modifications of the cysteine reaction which were described before. The reaction product of furfural and pentoses with cysteine is not stable under the conditions of these modifications, in which more diluted  $\text{H}_2\text{SO}_4$  is used. The reaction product of hexoses, for example, is unstable under the conditions of the modification of the cysteine reaction described before as CyR<sub>10</sub> (4). Only the products from methylpentoses can be observed at the end of the heating period in the latter reaction, and this disappears after further addition of water.

All classes of sugars, however, form other reaction products of characteristic color in these various forms of the cysteine reaction, namely the red compound of pentoses, the blue compound of hexoses (4), the yellow compound of methylpentoses (4), the purple compound of glucuronic acid

(2), and the blue compound of galacturonic acid (3), which develop only slowly after the addition of cysteine. They are formed only under conditions under which the reaction products of BCyR of these sugars are not stable and disappear. If 10 minutes after addition of cysteine, 0.6 cc. of  $H_2O$  is added to the reaction mixture of BCyR so that the final concentration of  $H_2O$  is brought to the same as in the modification designated CyR<sub>3</sub> (4), pentoses and furfural show, after 30 minutes, the red compound, and hexoses, after 24 hours, the characteristic blue compounds which are formed by those classes of sugars in CyR<sub>3</sub> after the same lapse of time. The variations in the amounts of the blue compound with different hexoses are about the same as in CyR<sub>3</sub>. The fact that furfural forms this secondary red compound indicates that its formation from pentoses in CyR<sub>3</sub> is due to the breakdown of the reaction product of furfural with cysteine in BCyR.

While the mechanism of BCyR of pentoses, methylpentoses, and hexoses appears to be very similar, that of 2-desoxypentoses differs considerably. No primary breakdown product in  $H_2SO_4$  absorbing between 320 and 350  $m\mu$  can be observed. No furfural, therefore, can be formed from these sugars under the conditions of BCyR. The reaction product with cysteine with a maximum at 375  $m\mu$  is formed slowly during 48 hours and its formation is not accelerated, as in the case of methylpentoses, by dilution with water. Experiments on furfuryl alcohol solutions showed that this compound behaves in BCyR exactly in the same way as does desoxyribose. The reaction product is formed slowly during 48 hours at room temperature, and its absorption curve shows a sharp maximum at 375  $m\mu$  and is in shape identical with that of desoxyribose. Furfuryl alcohol can be derived from 2-desoxypentofuranose by dehydration between carbon atoms 1 and 2 and carbons 3 and 4. No initial rearrangement is necessary for this reaction, as must be assumed for the formation of furfural from pentoses and its homologues from methylpentoses and hexoses. This can explain why only 2-desoxypentoses react with cysteine under the mild conditions of the specific modification of the cysteine reaction described previously (1).

This hypothetical mechanism of cysteine reaction is able to explain the characteristic differences in the behavior of various polysaccharides in BCyR. In the formation of furfural and its homologues from sugars under the influence of acids, we must assume a shift of the butylene oxide ring from the position 1,4 to positions 2,5. As long as the latter positions are not occupied by glycosidic linkages, the formation of furfural from the sugar may go on as if the sugar were free. If, however, one of carbons 2 and 5 participates in a glycosidic linkage, the reaction of the polysaccharide may go on in a very different way from that of the free sugar and formation of furfural may become possible only after prior hydrolysis of this

linkage. This view is supported by the facts that adenosine-5-phosphate reacts much more weakly in BCyR than adenosine-3-phosphate and, on the other hand, 2,5-anhydrohexoses give a much stronger BCyR than corresponding hexoses. From this it can be deduced that those polysaccharides which contain only 1,4 and 1,6 linkages, like amylose, dextran, and glycogen, will react as free glucose. On the other hand the position of the linkages by which the molecules of fucose in the blood group substances are linked to each other and to the glucosamine galactoside are not known completely, and it may be that carbons 2 and 5 are partly involved. In this case, methylfurfural will, therefore, be formed only after prior hydrolysis of these linkages. Kabat *et al.* (12) have recently shown that fucose can be split off the molecule of blood group substances by mild hydrolysis. Under relatively mild conditions of the BCyR, 1 part of fucose may be split off and form methylfurfural, while the part which is not split off will give other reaction products. This would explain the decrease in the value of  $D_{400}$  shown by the two blood group substances as compared with free fucose. In the other forms of the cysteine reaction of methylpentoses the heating is more intense and lasts longer. In this case nearly complete hydrolysis may occur before cysteine is added.

The basic cysteine reaction of sugars promises to be of practical significance in several respects. (1) It allows the simple and rapid detection on one sample of the classes of sugars present in an unknown solution, and can be, therefore, used as a preliminary step in any sugar analysis. (2) BCyR permits one to differentiate between ribose nucleotides substituted in position 5 and other nucleotides or pentoses. Dische and Schwartz (9) showed in 1937 that adenosine-5-phosphate gives a much more intense orcinol reaction than other nucleotides and nucleosides of ribose when the reaction mixture is heated only 3 minutes at 100°. Our more recent experiments (13) show that these differences disappear when the heating is continued for 20 minutes as reported first by Meijbaum (14) for the orcinol reaction carried out at lower concentrations of acid. By combining the two modifications of the orcinol reaction with BCyR it is, therefore, possible to determine various types of nucleotides in the presence of unidentified hexoses, as is the case in tissue extracts. Furthermore it was shown that coenzymes I and II behave differently from adenosine-5-phosphate in the orcinol reaction (13). Their presence requires the use of a third differentiating reaction for complete analysis. BCyR is an appropriate reaction for this purpose, as will be shown later. (3) It is possible to determine ribose nucleic acid in the presence of desoxyribose nucleic acid and hexoses. (4) Finally BCyR seems to be sensitive to specific linkages in polysaccharides and may enable us to differentiate between closely related polysaccharides of unknown structure. Blood group substances as



A and O from hog mucosa, which previously could be distinguished only by immunological reactions, give an example of such a differentiation.

#### SUMMARY

1. The breakdown products of sugars in a mixture of 1 volume of  $H_2O$  to 4 volumes of  $H_2SO_4$  combine with cysteine at room temperature to form products which show absorption curves with peaks varying between 375 and 410  $m\mu$  for different classes of sugars.

2. The velocity of the reaction with cysteine (BCyR) and the sensitivity of the final reaction products against water are different with different classes of sugars.

3. BCyR makes it possible to detect 10  $\gamma$  of methylpentoses, pentoses, and hexoses in mixtures in a single sample of 1 cc.

4. The application of BCyR to the quantitative determination of nucleotides and ribose and desoxyribonucleic acids in mixtures with each other and with hexoses is discussed.

5. The nature of the primary breakdown products of sugars in  $H_2SO_4$ , the general mechanism of BCyR, and other modifications of the cysteine reaction of carbohydrates are discussed.

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# NEOPYRITHIAMINE AND THE THIAMINASE OF FISH TISSUES\*

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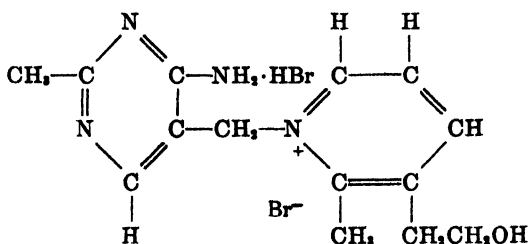
The enzyme of the visceral tissues of carp, mullet, and other fishes which destroys thiamine by cleaving the pyrimidylmethylene group from the thiazole portion may be either inhibited or activated by appropriate analogues of the vitamin. Sealock and Goodland (1) have shown that 3-(*o*-aminobenzyl)-4-methylthiazolium chloride specifically and competitively inhibits the enzymatic destruction of thiamine. On the other hand, when the amino group is present in the meta instead of the ortho position, the corresponding compound, 3-(*m*-aminobenzyl)-4-methylthiazolium chloride fails to inhibit the enzyme. Instead activation is observed (2). Incubation of the enzyme and thiamine with this analogue results in very significantly increased destruction of the thiamine. As a result of these findings it has been suggested (2) that the position of the amino group relative to the methylene attachment to the thiazole ring is of utmost importance to the process of inhibition. In fact it would appear that both the inhibitor and the substrate combine with the enzyme by means of their respective amino groups (1, 2). This suggestion became even more valid when it was found that the activation by the *m*-amino compound or a substitute for it, *m*-nitroaniline (or *m*-aminobenzoic acid), resulted from an entirely different mechanism (3). These latter compounds produce their activation by serving as "reactors" during the enzymatic destruction of thiamine, being converted into secondary amino derivatives with the pyrimidylmethylene residue.

That the amino group is not the only requisite to an effective inhibitor may also be concluded, since *o*-aminobenzyl alcohol does not inhibit the enzyme and the aminopyrimidylmethylenesulfonic acid derived from thiamine is a very poor inhibitor (1). It is quite conceivable that the latter compound, if present in the form of an aminopyrimidylmethylene quaternary salt rather than a sulfonic acid, would then become a more effective inhibitor. Such a compound is available in neopyrithiamine or 1-((4-amino - 2 - methyl) - 5 - pyrimidylmethyl) - 2 - methyl - 3 - ( $\beta$  - hydroxyethyl)-pyridinium bromide hydrobromide (I), the isostere of thiamine

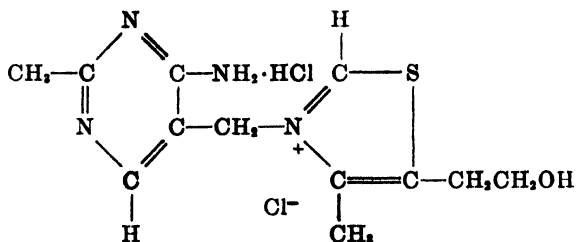
\* The authors are indebted to Dr. Karl Folkers, Director of Organic and Biochemical Research of the Research and Development Division of Merck and Company, Inc., for making available a supply of neopyrithiamine and to the Williams-Waterman Fund of the Research Corporation for a grant-in-aid in support of this investigation.

(II) described by Wilson and Harris (4) and which should be distinguished from the better known pyriethamine of Tracy and Elderfield (5). The latter was originally supposed to be the above isostere of thiamine but according to the report from the Merck laboratories (4) is a mixture of polymeric substances, none of which possess the correct structure.

Thiamine was incubated with the enzyme under the usual conditions (1) including 0.63, 1.25, and 2.5  $\mu\text{M}$  of neopyrithiamine in appropriate tubes. At the conclusion of the incubation the analysis of thiamine by the Melnick and Field procedure (6) showed that 61 per cent of the thiamine had been destroyed by the enzyme in the absence of neopyrithiamine.



(I)



(II)

With the compound present there was less destruction of thiamine. As may be seen in Table I, this decreased destruction amounts to calculated inhibitions of 19, 27, and 40 per cent, respectively, with the three concentrations of neopyrithiamine employed. With a different enzyme preparation, similar results were obtained, the calculated inhibition amounting to 39.9 per cent, as may be seen in the lower portion of Table I. Entirely similar results from additional experiments have been omitted for the sake of brevity. From these experiments it would be concluded that the thiamine isostere inhibits the destruction of the vitamin by the fish enzyme. A more critical examination of the reaction has shown that such a conclusion does not entirely describe the actual happenings, as will be shown in later paragraphs.

In order to use the Melnick and Field method of analysis in the above experiment, it was of course necessary to show that neopyrithiamine neither interfered nor reacted to give xylene-extractable chromogenic material. Under the same conditions employed for thiamine analysis, neopyrithiamine gave no color value even when the reagent was allowed to react for 0.5, 6, and 24 hours before adding the xylene. At the same time, the presence of neopyrithiamine had no effect on the thiamine color value.

It is of interest to point out that this failure of neopyrithiamine to react in the Melnick and Field method furnishes strong evidence relative to the nature of the reaction obtained with diazotized *p*-aminoacetophenone and thiamine. The presence of the alkali in the reagent causes the opening of the thiazole ring and formation of the sulfhydryl group, as is well known

TABLE I  
*Apparent Inhibition of Thiaminase with Neopyrithiamine*

The incubations were carried out in 5 ml. of 0.08 M phosphate buffer and 4 per cent sodium chloride at pH 7.4 and 37.5° for 2 hours. The enzyme was the soluble portion from the amount and preparation indicated. 2.5  $\mu$ M of thiamine ( $5 \times 10^{-4}$  mole per liter) were present in each tube.

	Neopyrithiamine	Thiamine destroyed	Inhibition
	<i>moles per l. <math>\times 10^4</math></i>	$\mu$ M	<i>per cent</i>
Enzyme D1-215, 40 mg. equivalents per tube	0	1.58	
	1.25	1.22	19.1
	2.50	1.11	26.5
	5.00	0.92	39.6
Enzyme S3-110, 60 mg. equivalents per tube	0	0.98	
	5.0	0.59	39.9

(7). If we assume that the Melnick and Field reaction involves coupling of the diazo reagent with the sulfhydryl group in order to form the colored (xylene-soluble) complex, it is obvious that neopyrithiamine, which neither contains sulfur nor can undergo analogous pyridine ring opening, should not react in this method. While considerable evidence has accumulated in this laboratory that this is the mechanism of the reaction, a more complete description will be presented later.

The lack of interference of neopyrithiamine in the analytical method has permitted the calculation of apparent inhibition in the experiment recorded in Table I. However, the similarity in structure of the compounds is such that the enzyme might be expected to produce the same cleavage of neopyrithiamine that it does of thiamine. In order to test this possibility it is necessary to have a method of analysis for the former. The possibility that it would undergo the alkaline oxidation and subse-

quent production of fluorescence as does thiamine in the thiochrome procedure (8) was therefore tested. Solutions of neopyrithiamine, such as were used in the enzyme experiments, containing  $2.5 \mu\text{M}$  in 10 ml. of 0.04 M phosphate buffer and 5 per cent trichloroacetic acid, were diluted with water. 5 ml. of a 1:250 dilution treated with 3 ml. of alkaline ferricyanide and read directly without butyl alcohol extraction gave galvanometer readings of 11 (corrected for the blank) which correspond to the readings obtained with approximately one-fifth the quantity of thiamine. With dilutions of 1:50, scale readings of 75 were obtained and it became obvious that the new compound could be determined by means of

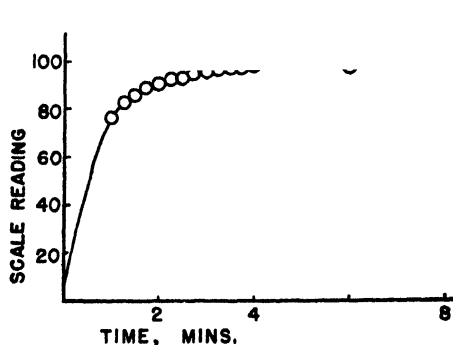


Fig. 1

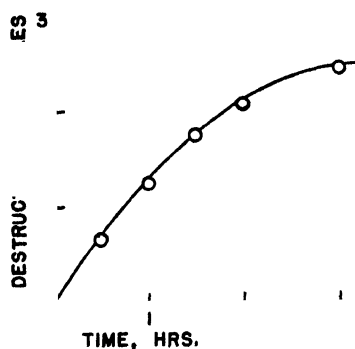


Fig. 2

FIG. 1. The development of fluorescence with neopyrithiamine in the thiamine-thiochrome procedure.  $0.025 \mu\text{M}$  of neopyrithiamine in 5 ml. of solution was treated with alkaline ferricyanide and the readings obtained directly with the reaction mixture.

FIG. 2. The destruction of neopyrithiamine by carp thiaminase measured at different time intervals.  $2.5 \mu\text{M}$  of neopyrithiamine in 5 ml. of reaction mixture were incubated with the enzyme in the usual fashion, the soluble portion from 50 mg. of acetone-desiccated powder being used in each 5 ml.

the thiochrome procedure. At concentrations of 0 to  $0.02 \mu\text{M}$  per 5 ml. of aliquot analyzed the galvanometer readings were found to be proportional to the concentration. With this method 5.70 moles of neopyrithiamine yield fluorescence equivalent to 1 mole of thiamine. All readings have been taken at 90 seconds after addition of the reagent, although more recently it has been found that maximum fluorescence is not developed in this length of time. Analysis of this factor has led to the results in Fig. 1 which shows that the maximum value is obtained at approximately 4 minutes. The curve likewise shows that the 90 second interval used in these experiments is beyond the time of rapid reaction, so that probably no great error was introduced. The fluorescence from neopyrithiamine does not appear in the absence of ferricyanide (analytical control) and

may be extracted with isobutanol as in the usual thiochrome procedure. However, this latter step was not necessary in these experiments, because of the relatively high concentration of compound employed. Neopyrithiamine may also be removed from solution by Decalso, as employed in the more complete thiochrome procedure, and elution may be accomplished in the usual fashion, although this likewise proved unnecessary in the experiments described here.

With the possibility of determining neopyrithiamine established, this compound was incubated with the enzyme under the same conditions employed with thiamine. In each tube the soluble material from 40 mg.

TABLE II  
*Destruction of Neopyrithiamine by Thiaminase*

The incubations were carried out as described in Table I except for the modifications indicated and for the substitution of neopyrithiamine in the amounts indicated for thiamine.

Experiment No.	Enzyme present	Neopyrithiamine		
		Concentration	Destruction	
		moles per l. $\times 10^4$	$\mu M$	per cent
I	None	2.50	0.02	1.6
	"	5.00	0.03	1.2
	D1-215, 40 mg. equivalents	1.25	0.60	96.2
	D1-215, 40 "	2.50	1.20	96.0
	D1-215, 40 "	5.00	1.44	58.0
II	D1-215, 40 " equivalents	2.5	1.25	100.0
	D1-215, 40 "	5.0	1.52	60.8
	Same, boiled	2.5	0.04	3.2
	" "	5.0	0.14	5.7

of acetone-desiccated powder was used as the source of the enzyme. Neopyrithiamine at the levels of 0.63, 1.25, and 2.5  $\mu M$  was added to appropriate tubes. At the end of the 2 hour incubation period, the neopyrithiamine remaining was determined. At the two lower levels of compound almost complete destruction occurred, as may be seen in Table II. At the higher level only 57.6 per cent was destroyed. Thus the reaction in this respect is similar to the thiamine reaction. As also may be seen in Table II incubation of the compound in the buffer solution alone caused no appreciable destruction. In the second experiment shown in Table II incubation of 1.25 and 2.5  $\mu M$  of neopyrithiamine with the same amount of enzyme preparation resulted in 100 and 60.8 per cent destruction respectively. In contrast, incubation with an equivalent amount of previously boiled enzyme extract produced essentially no destruction of the added neo-

pyrithiamine. Additional experiments have yielded similar results; however, the enzymatic nature of the reaction is even more evident in the graph of neopyrithiamine destruction with time, shown in Fig. 2. The curve obtained is typical of enzyme reactions and in fact resembles closely the analogous curve obtained with thiamine (9).

With these results it may then be concluded that neopyrithiamine sufficiently resembles thiamine in structure so that it too can undergo destructive reaction by the fish enzyme. It also may be concluded that the apparent inhibition recorded in Table I is not inhibition but instead is a case of enzymatic action in the presence of competitive substrates. Each of the substrates when present in the same enzyme solution is competing with the other for the enzyme. Confirmation of this conclusion has been obtained in experiments in which the enzymatic destruction of each substrate alone has been compared with that obtained when the two are present in the same solution. In such an experiment thiamine destruction is determined by the Melnick and Field procedure, whereas the total neopyrithiamine and thiamine destruction is measured by the thiochrome method. By deducting the former value from the latter the amount of neopyrithiamine destroyed was then calculated. When  $2.5\ \mu\text{M}$  of each compound were employed with the particular enzyme preparation used, analytical difficulties were encountered due to the unfavorable ratio in chromogenic value in the fluorescence method. Consequently a precise evaluation of the neopyrithiamine destruction was difficult. By employing 4 times as much neopyrithiamine as thiamine a more favorable ratio was obtained for analytical purposes. With  $5.0\ \mu\text{M}$  of the former and  $1.25\ \mu\text{M}$  of the latter it was found that simultaneous destruction of each compound occurred, as may be seen in Table III.

It should be pointed out also that neopyrithiamine is only the second substrate, even though an artificial one, to be discovered for this enzyme. We, of course, may discount cocarboxylase, even though it is also destroyed, since it is merely the pyrophosphate ester of the original substrate. None of the other thiamine analogues which have been studied have been found to undergo the enzymatic reaction, although some of them are excellent competitive inhibitors.

The results obtained in this study permit at least tentative conclusions relative to one aspect of the mechanism of the thiaminase action. Since it is almost certain that neopyrithiamine is incapable of undergoing the ring opening characteristic of the thiamine-thiazole ring in alkaline solution, it may be argued that the enzymatic destruction of neopyrithiamine occurred with the molecule in the form indicated on a previous page. If the enzyme is able to produce its characteristic reaction with neopyrithiamine, which is incapable of ring opening, then one may tentatively con-

clude that the enzyme reaction in the case of thiamine is entirely analogous. Or in other words, we would suggest that the thiaminase action involves a cleavage of the linkage between the two ring units of thiamine when the thiazole moiety is present in the intact and closed ring form. This conclusion is also supported by the fact that the intact thiazole unit can be recovered from the enzymatic reaction as a product of the reaction.

If the conclusions at this point are correct, then it would be predicted that the compounds which specifically inhibit the destruction of thiamine by the fish thiaminase would also inhibit the destruction of neopyrithiamine. Likewise, compounds producing activation of thiamine destruction would have a similar effect upon neopyrithiamine destruction. Preliminary ex-

TABLE III

*Simultaneous Destruction of Thiamine and Neopyrithiamine*

The incubations were carried out as described in Table I except for the modifications indicated. The enzyme preparation in each tube represented the soluble portion from 33 mg. of Enzyme S3-138. Thiamine was determined by the Melnick-Field procedure and neopyrithiamine by subtracting the Melnick-Field value from the total fluorophotometric values.

Compound present	Concentration <i>moles per l. <math>\times 10^4</math></i>	Compound destroyed	
		$\mu\text{M}$	<i>per cent</i>
Single substrates			
Thiamine . . . . .	2.5	0.792	63.3
Neopyrithiamine.	10.0	0.860	17.2
Combined substrates			
Thiamine . . .	2.5	0.190	15.2
Neopyrithiamine	10.0	0.870	17.4

periments in this direction entirely confirmed both predictions.  $\beta$ -Aminoethyl-4-methylthiazolium chloride, previously shown to be an excellent inhibitor of this enzyme (1), was found to inhibit neopyrithiamine destruction, and *m*-nitroaniline (3) caused an increased enzymatic destruction of neopyrithiamine. Thus additional evidence for identity of the enzyme producing destruction in the case of the two substrates is obtained.

In conjunction with the analyses made in the enzymatic experiments, the ultraviolet absorption spectra of neopyrithiamine were studied. Even though it is a 6-aminopyrimidine derivative and accordingly absorbs ultraviolet light as thiamine, there are sufficient differences so that each of the two compounds may be quantitatively determined in the same solution. However, it is necessary to maintain control of the pH. For example, in 2.5 *N* hydrochloric acid and at pH 0.45 and 1.25, neopyrithiamine exhibits two maxima, whereas thiamine exhibits only a single



maximum at acidities greater than pH 5.0. For the former compound the maxima are to be found at 2440 and 2660 Å at pH 4.0. These values are essentially in agreement with those recorded by Wilson and Harris (4), although the solvent and pH were not stated in their case. A mini-

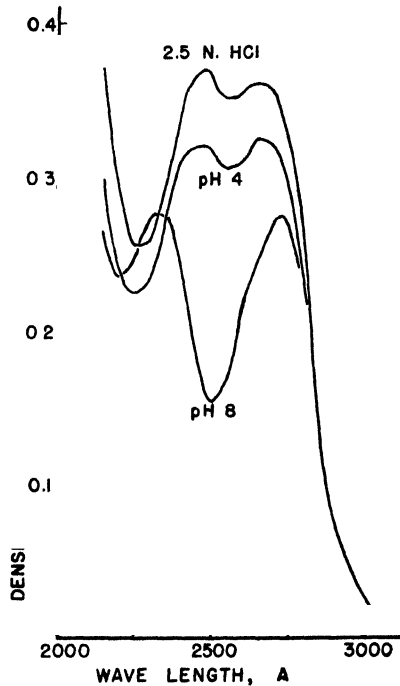


FIG. 3

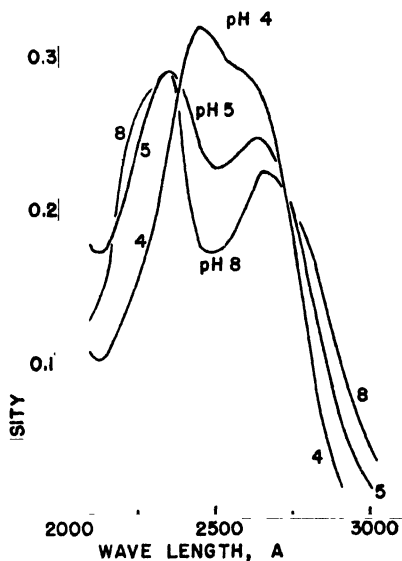


FIG. 4

FIG. 3. The ultraviolet absorption spectra of neopyrithiamine in aqueous solution and at different acidities. The compound was present in  $2.5 \times 10^{-5}$  M concentration. At pH 4 and pH 8, 0.04 M phosphate buffer was present. The Beckman spectrophotometer, model DU, with 1 cm. cells was employed. The instrument was adjusted with the solvent as the blank, and a minimum slit width was used at all wave-lengths. Density =  $\log (I_0/I)$ .

FIG. 4. The ultraviolet absorption spectra of thiamine ( $2.5 \times 10^{-5}$  M) in aqueous solution and at different acidities. 0.04 M phosphate buffer was present in each solution.

um is also present at 2570 Å, as may be seen in Fig. 3. At pH 5 and higher the two compounds are more nearly alike in their ultraviolet absorption, as is evident from a comparison of Figs. 3 and 4. Since the shift from a single maximum to the two maxima is usually characteristic of the 6-aminopyrimidine compounds as one proceeds from acid to more alkaline solution, both compounds would be expected to exhibit this phenomenon. However, it is evident that the occurrence of this shift is dependent upon

the substituent attached to the 5-methylene group of the pyrimidine ring. When this shift does occur with typical 6-aminopyrimidine compounds, it has been observed that the pH at which the two maxima appear is a characteristic of the individual compound, and it even has been possible to distinguish and identify some of the different 6-aminopyrimidines by the pH value and other aspects of the shift.<sup>1</sup> This in turn emphasizes the necessity of control of pH in determining the absorption spectra of such compounds.

In the face of the findings with neopyrithiamine described above it is of interest to summarize briefly and illustrate the present status of thiamine with regard to its analogues. Thiamine is somewhat unique among biologically important compounds. On the one hand, there is the rôle of this substance as an important vitamin and accessory factor producing physiological effects, primarily through the enzymatic functioning of its pyrophosphate ester, cocarboxylase. This rôle may be designated as that of physiological function. On the other hand, there is the destruction of thiamine by an enzyme present in a number of unrelated species, and at the moment, it is hardly conceivable that the latter phenomenon could be regarded as a component of the former. For each category there are one or more specific analogues that may be employed for the purpose of further analysis of mechanisms involved or useful inhibition. Neopyrithiamine is an antagonist to thiamine function (4), as is pyrithiamine (10), and is also a substrate for the fish enzyme. In contrast, the *o*-aminobenzylthiazolium compounds inhibit the fish enzyme (1), but show no antagonism toward thiamine function.<sup>2</sup> The corresponding aminoethyl- and aminopropylthiazolium analogues exhibit similar behavior, being relatively strong inhibitors of the enzymatic destruction of thiamine. With the *m*-aminobenzylthiazolium compounds neither antagonism to thiamine function nor inhibition of destruction is observed. Instead, activation of the destructive process is obtained. All of these compounds represent extensive modification of the molecular composition of thiamine and in no instance do we find them able to substitute for the vitamin in its physiological function. However, with only slight modification active substitutes are obtained. For example, the 2-ethylpyrimidine analogue of thiamine possesses physiological activity (11). Without referring to numerous other interesting analogues which have been studied in more or less detail, it is obvious that a number of structural modifications are available for use in determining the biochemical properties and behavior of thiamine. In particular, they should make possible a most detailed analysis of the fundamental aspects of enzyme relationships of thiamine. The fact that

<sup>1</sup> Sealock, R. R., and White, H. S., unpublished data.

<sup>2</sup> Sealock, R. R., and Picken, J. C., unpublished data.

they may also have practical uses yet to be discovered only emphasizes the interest in their further study.

#### EXPERIMENTAL

The thiaminase used in these experiments was prepared from acetone-desiccated powders of carp viscera (9) by extracting with 10 per cent sodium chloride in 0.2 M phosphate buffer at pH 7.4. The combined extracts were diluted with the solvent so that 2 ml. contained the soluble portion of the indicated amounts of the powder and were adjusted to pH 7.4 before being used. 2 ml. of this extract plus the desired volumes of substrates and inhibitors or activators were placed in test-tubes and when necessary the volume made to 5 ml. with water. The tubes were incubated for 2 hours at 37.5°, and unless otherwise indicated, the reaction was stopped by the addition of 5 ml. of 10 per cent trichloroacetic acid. After at least 30 minutes the precipitated protein was removed by filtration. For thiamine determination 2 ml. aliquots of the filtrates were analyzed by the Melnick-Field method. For determination of neopyrithiamine 5 ml. aliquots of a solution made by diluting the filtrates 1:50 were treated with 3 ml. of an alkaline ferricyanide solution (3 ml. of 1 per cent potassium ferricyanide diluted to 100 ml. with 15 per cent sodium hydroxide), or, in order to determine the blank galvanometer reading, with 3 ml. of 15 per cent sodium hydroxide. In determining the total fluorescence in the presence of both substrates, 2 ml. aliquots of the filtrates were diluted to 250 ml., and 5 ml. aliquots analyzed as above. The fluorophotometer was adjusted with a quinine solution to allow maximum use of the galvanometer scale. In all analyses comparison was made with unincubated controls to which trichloroacetic acid was added immediately after the addition of the substrate.

#### SUMMARY

In preliminary experiments neopyrithiamine was found to inhibit the destruction of thiamine by the thiaminase of carp tissue.

The calculated inhibition produced by neopyrithiamine proved not to be inhibition in the usual sense, for this analogue in turn undergoes the same destructive reaction with thiaminase as does thiamine. Neopyrithiamine therefore possesses in its isosteric structure the necessary components for complete action of the enzyme. It is therefore an additional substrate for this enzyme. When the two compounds are present in the same enzyme solution, they may be regarded as competing substrates.

Neopyrithiamine neither reacts nor interferes in the Melnick and Field diazotized *p*-aminoacetophenone method for the determination of thiamine.

It does, however, react under the conditions of the thiochrome procedure and may be quantitatively determined by this method.

With appropriate control of pH, thiamine and neopyrithiamine may be readily distinguished by means of their ultraviolet absorption spectra, for the analogue exhibits two maxima in acid solution in contrast to thiamine which exhibits only one maximum at acidities less than pH 5.

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# ELECTROPHORETIC AND ULTRACENTRIFUGAL ANALYSES OF PROTEIN EXTRACTED FROM WHOLE MAMMALIAN MUSCLES

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In the extraction of tissues and organs, the present practice is to grind, mince, or homogenize. Another technique is, however, possible, when speed is not essential. It consists in the slower removal of soluble material from whole organs, or major parts thereof. Such a procedure may properly be called "mild extraction." It was used earlier by Luckhardt, Barlow, and Weaver (1) in their removal of secretin from the gut wall. A similar technique has sometimes been used to extract intestinal enzymes. It has rarely been applied to other tissues.

We adopted mild extraction in order to combine histological and biochemical techniques in a study of the proteins of skeletal muscle. Specifically, we wished to discover the location of the several known proteins within the sarcomere. We hoped to preserve the structure, so far as possible, as we removed the proteins seriatim, using conventional extraction media. We arranged to freeze and thaw the muscles before extraction. Such treatment expedites the egress of protein from the whole tissues, but greatly changes the histological picture, even before extraction begins, as we have recently discovered.

In spite of this failure to attain our original objective, we find that the freezing technique yields protein solutions which have considerable biochemical interest. They do not show the turbidity usually found in extracts of muscle and other tissues, and they yield electrophoretic and ultracentrifugal patterns which are clear and definite, with satisfactory consistency in the calculated mobilities.

Most previous electrophoretic studies of muscle extracts have been made with more or less purified solutions of individual proteins (2-5). Mehl and Sexton (6) have published a brief summary of work carried out with extracts of rat and octopus muscle, but give no patterns or mobility values. Only in the Liège laboratory have systematic attempts been made to study simultaneously the whole range of components found in unfractionated extracts of minced muscles, at various pH values and ionic strengths (7-10). As many as eleven components have been described in such extracts. The

exact relationships existing between these components and the classical muscle proteins have not been established.

Our present communication is concerned with the electrophoretic and ultracentrifugal patterns obtained from extracts of muscles made within the 1st week after excision of the tissues. The results are offered as a suggestion to other investigators that protein solutions obtained by mild extraction may have a rôle in the further development of the study of muscle. Preliminary reports have been published (11, 12).

### *Methods*

In order to remove blood from the tissues, our animals, which in all cases have been rabbits, were exsanguinated. Oxygenated Ringer-Locke solution is infused through a cannula, placed in the jugular vein, at the same rate the blood is withdrawn from the carotid artery. In this way, almost all of the blood (96 to 98 per cent) may be washed out before the heart fails.

Two distinct types of striated muscle fibers exist. One type contains myoglobin and is red in color; a second type lacks this pigment and is nearly white. A few muscles consist exclusively of one or the other type; most muscles contain mixtures of the two. The differences in color are accentuated after nearly total exsanguination. We have used deep red muscles, such as soleus, semitendinosus, and vastus intermedius, and nearly white muscles, such as adductor longus and brevis and adductor magnus. Myoglobin may be quantitatively extracted from whole red muscles, forming a wine-red solution. White muscle extracts are practically colorless.

The separate muscles were carefully removed and transferred to weighing bottles with ground glass covers. The bottles were partly immersed in a bath of carbon dioxide snow and alcohol until the tissues were solidly frozen. The bottles were then removed, dried, and weighed. After being thawed, the muscles were transferred to small flasks to which were added 0.4 M potassium phosphate buffer solutions at pH 7.6. From 1 to 2 cc. of solution was added per gm. of muscle, the exact volume being known in each case. Extraction proceeded in the cold room at 0°. For many days, such buffer solutions remove only elements of the myogen complex, including myoglobin. In order to remove myosin as well, sodium pyrophosphate was added in the amount of 500 mg. per cent.

In preparation for electrophoresis, the solutions were poured off, centrifuged once to remove tissue débris, and usually dialyzed against 0.1 M potassium phosphate buffer solution at pH 7.6. In a few cases, 0.05 M buffers have been used, but they were not satisfactory, since myosin partially precipitates. All extractions and dialyses were carried out without adding calcium or magnesium salts. Total protein in the dialyzed extracts

was determined by a refractometer. Viscosity measurements were made on the same dialyzed extracts. Pyrophosphate, added to extract myosin, was reduced to very low concentration (<3 mg. per cent) during dialysis. Electrophoretic determinations were made at 0°. They were carried out at Upsala with the Philpot-Svensson modification of the Tiselius technique. The sedimentation diagrams were also obtained at Upsala with the Svedberg ultracentrifuge.

### \* Results

*Electrophoretic Patterns*—It was our original expectation that 0.4 M potassium phosphate buffer solutions would be able to extract myosin readily from whole muscles, since they are able to dissolve this protein after its extraction. Such was not the case. The viscosity of the extracts was only slightly higher than that of their corresponding buffers. The addition of actin caused no rise in viscosity; neither did adenosine triphosphate reduce it. Electrophoretic patterns were simple, showing no component which could be identified as myosin. In white muscle extracts, only two components, A and D, were visible. In red muscle extracts, three components could be seen, A, B, and D. Examples of both patterns are shown in Fig. 1, Section I. These same components, in lower concentration, could be extracted by distilled water, and must be considered fractions of the myogen complex. In 0.4 M phosphate buffer solutions, the protein extracted within the 1st week amounts to approximately 1 per cent of the wet weight of the muscle. The percentages vary between 0.6 and 1.6.

It thus became clear that there is a *structural factor* in the solubility of muscle proteins. Solutions which readily extract myosin after the structural disintegration of the tissue are unable to dissolve it within the whole muscle mass. Dr. Hans Hoch suggested the use of adenosine triphosphate, on the hypothesis that it might dissolve the link between myosin and actin within the fibrils, just as it does in actomyosin solutions. Myosin might thus be set free to diffuse from the muscle. Since adenosine triphosphate would have been quickly split by muscle adenosinetriphosphatase, we decided to make use of the discovery of Straub (13) that pyrophosphate, alone among inorganic phosphates, can exert an adenosine triphosphate-like activity, at temperatures at or near 0°, without being broken down by enzyme action.

Accordingly we began to add sodium pyrophosphate to some of our extracting solutions, in the same concentration which Straub originally used. Such extracts were much more viscous and higher in protein content than those obtained without the use of pyrophosphate. Addition of adenosine triphosphate gave no significant change in viscosity, indicating the absence of actomyosin. The addition of actin solution, on the other hand, caused



a considerable rise in viscosity, which disappeared when adenosine triphosphate was applied. It now seemed certain that pyrophosphate is indeed able to dissolve the link between actin and myosin, so that the latter can diffuse.

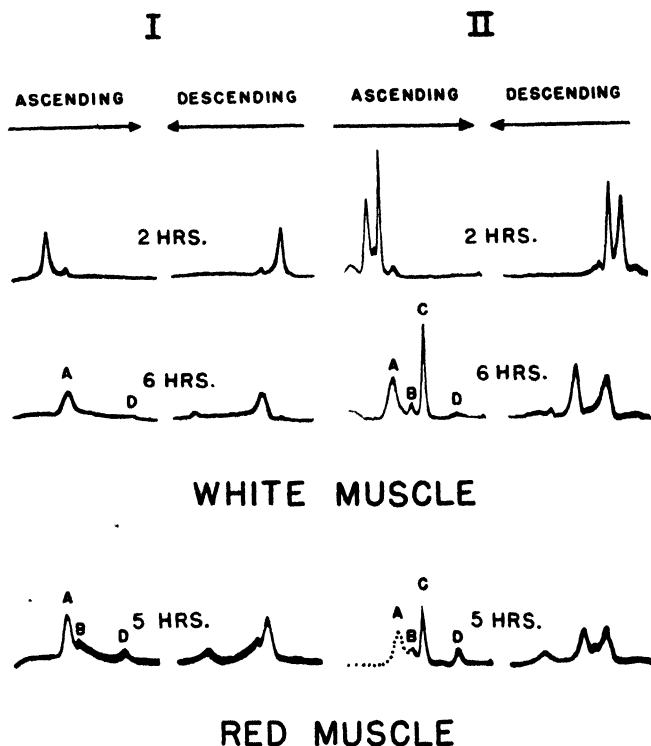


FIG. 1. Influence of pyrophosphate upon extraction of protein from skeletal muscles. In Section I are shown typical electrophoretic patterns secured from extracts of whole muscles, both white and red, made with 0.4 M potassium phosphate buffers at pH 7.6. In Section II are shown the patterns secured from similar extracts made with the addition of 500 mg. per cent of sodium pyrophosphate to the phosphate buffers. For further description see the text.

Electrophoretic analysis confirmed this conclusion. The patterns took the forms shown in Section II of Fig. 1. In extracts of white muscle, two new components, B and C, appeared. In red muscle extracts, a C component also appeared, with little or no change in the B component. We have retouched the plate in the ascending limb of the red muscle pattern, using dots to outline those portions of the pattern which were obscured by myoglobin. Complete photographs of the rest of the red muscle patterns were obtained by exposing the obscured areas much longer than the rest

of the record. The red color extends to the top of the B component, which is thus identified as myoglobin.

Table I presents a summary of mobilities calculated for both ascending and descending limbs in fifteen experiments. The two limbs gave nearly

TABLE I

*Electrophoretic Mobilities of Proteins Extracted from Whole Muscles in Phosphate Buffers at pH 7.6*

Averages		Mobility $\times 10^4$			
		Component A	Component B	Component C	Component D
15 experiments	Ascending	1.90 (1.68-2.26)	2.80 (2.59-3.10)	3.61 (3.33-3.86)	5.92 (4.51-7.49)
	Descending	1.90 (1.42-2.36)	2.76 (2.45-3.01)	3.39 (3.10-3.74)	6.00 (4.81-6.95)
In 0.10 M K phosphate					
11 experiments	Ascending	1.92 (1.75-2.26)	2.80 (2.59-3.10)	3.66 (3.33-3.86)	5.64 (4.51-6.12)
	Descending	1.94 (1.42-2.36)	2.79 (2.45-3.01)	3.39 (3.10-3.74)	5.67 (4.81-6.16)
2 white muscles	Ascending	1.88 (1.87-1.90)			5.01 (4.51-5.52)
	Descending	2.20 (2.04-2.36)			6.08 (6.01-6.16)
4 white muscles + pyrophosphate	Ascending	1.92 (1.75-2.26)	2.83 (2.62-3.10)	3.69 (3.33-3.86)	5.70 (5.25-6.12)
	Descending	1.83 (1.42-2.18)	2.89 (2.77-3.01)	3.31 (3.22-3.42)	5.43 (4.81-6.16)
1 red muscle	Ascending	1.96	2.59		5.85
	Descending	2.01	2.95		4.96
2 red muscles + pyrophosphate	Ascending	1.90 (1.88-1.93)	2.85 (2.72-2.98)	3.44 (3.42-3.47)	5.77 (5.65-5.90)
	Descending	*			
1 heart muscle	Ascending	1.94		3.76	5.90
	Descending	1.99		3.74	6.06
1 heart muscle + pyrophosphate	Ascending	1.91		3.42	5.90
	Descending	2.09		3.61	6.14

\* No readings made.

the same mobilities. Nine extracts were derived from white muscle, four from red muscle, and two from heart. In four of the experiments, 0.05 M potassium phosphate buffers were used, in eleven, 0.10 M buffers, all at pH 7.6.

Components A and D gave mobilities which were nearly the same for all

three types of muscle. Similarly, Component B had nearly the same mobility in both red and white muscle extracts. It is readily extracted from red muscles by phosphate buffers and is not notably increased in concentration by the addition of pyrophosphate. In extracts of white muscle, it appears only when pyrophosphate is used and is colorless. We cannot as yet identify it. Conceivably it is globin, not united with a colored prosthetic group.

Component C, in extracts of both red and white muscles, appears only when pyrophosphate has been added to the extracting solutions. Its behavior is characteristic of a viscous protein.

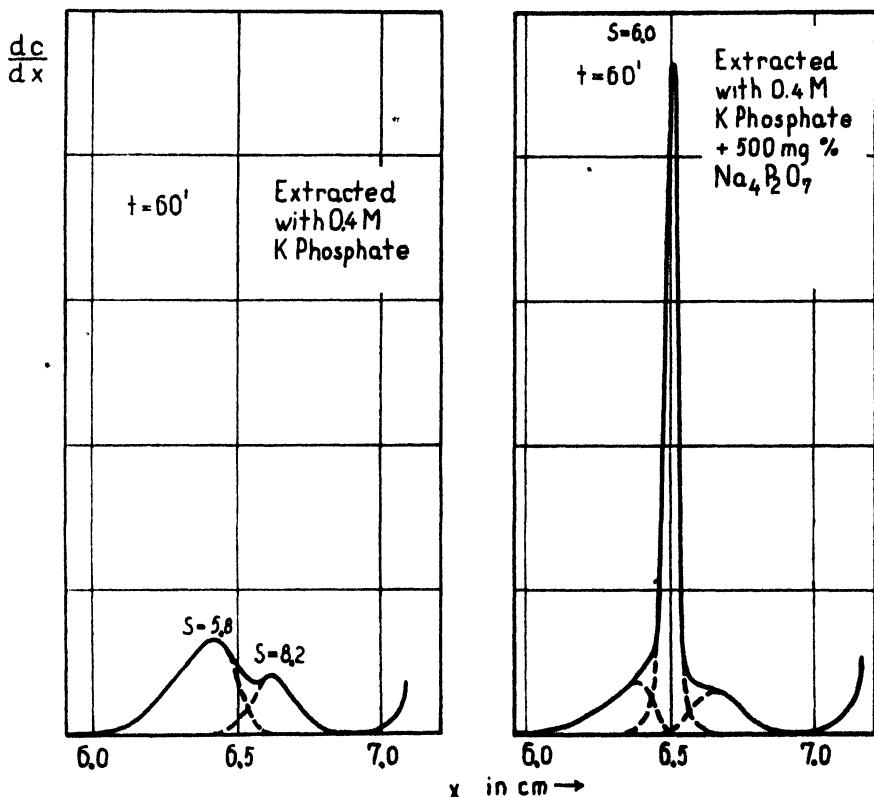
In order to establish the identity of Component C, we resorted to electrophoretic fractionation. In several tests with white muscle extracts, we have been able to separate Components B, C, and D from Component A. In the separated fraction, in which Component C comprised about 70 per cent of the protein present, the solution gave positive tests for myosin. In one longer fractionation of a red muscle extract, we have been able to separate Component C from all others, continuing electrophoresis until the fast component, D, had completely left the cell. The solution containing only Component C gave a positive test for myosin. Actomyosin could not be detected.

Our information concerning heart muscle extracts is incomplete. Myoglobin is present in heart muscle and is readily extracted, but we have not been able to identify it in the electrophoretic pattern or determine its mobility. A component with the mobility of myosin appeared in the one experiment in which no pyrophosphate had been added. In the companion run, in which extraction was made with the aid of pyrophosphate, this component became more prominent. We have accepted it as a C component. Tentatively, we may conclude that myosin is not strongly linked with actin in heart muscle, and hence is in part dislodged without the aid of pyrophosphate.

Our electrophoretic patterns do not give information concerning the relative amounts of the various components originally present in the muscle. They merely disclose the proportions present at a particular time as derived by the mild extraction technique.

*Sedimentation Diagrams*—We have obtained a few tests of extracts by the ultracentrifuge in Upsala. In Fig. 2, the results obtained with two extracts of white muscle are presented, one made with 0.4 M potassium phosphate buffer alone (pH 7.6), the other with the same buffer after addition of pyrophosphate. In the second diagram, myosin appeared as a sharply defined boundary which retained its discrete character throughout the whole run. The observations support our other results in demonstrating the ability of pyrophosphate to extract myosin from the whole muscle. The sedimentation constant of myosin at infinite dilution, calculated from

two runs with red muscle extract, was 7.4 S. In this experiment, the slope of the  $s$ - $c$  curve was determined by two readings; the value of  $s_{20}$  was 6.49 when the total protein concentration was 0.33 per cent, and 5.53 when it was 0.67 per cent. In a determination on a white muscle extract, the  $s_{20}$



### SEDIMENTATION DIAGRAM OF WHITE MUSCLE EXTRACTS

FIG. 2. The ordinates are the derivatives of protein concentration ( $c$ ) with respect to the distance from the axis of rotation ( $x$  in cm.). The abscissae are the distances from the axis of rotation ( $x$  in cm.).  $s$  values are sedimentation constants in Svedberg units.

value was 4.5 when the total protein concentration was 1.32 per cent, and 6.04 when the total protein concentration was 0.43 per cent. The  $s_{20}$  value at infinite dilution in this second case was calculated to be 6.8. Available data are not sufficient to give a precise determination of the  $s$  value at infinite dilution. The two figures are in fair agreement with the value of 7.2 S, recently determined for myosin extracted from minced muscles by Snellman and Erdős (14).

## DISCUSSION

These observations establish the validity of mild extraction as a procedure adapted to the removal of some of the protein from whole muscles after they have been frozen and thawed. It is not surprising that myoglobin, with molecular weight of 17,200 (15), should readily diffuse from the muscle mass. It was less to be expected that larger myogen fractions with molecular weights from 80,000 to 150,000 (16, 17) would also diffuse. The diffusion of the structural proteins seemed even less probable. Estimates of the molecular weight of myosin in the literature range from 1,000,000 to 4,000,000 (4, 14, 18). If so large a molecule were globular, its diffusion through the muscle mass might indeed be impossible. Molecules of the structural proteins, however, are long thin fibers (19), whose form may permit them to move slowly through the membranes and other barriers within the tissues. Certainly our experimental evidence demonstrates that appreciable fractions of myosin can diffuse out of the whole muscle after its union with actin within the fibrils has been broken by pyrophosphate.

It is hazardous to attempt to relate the four components described here with the more numerous family of elevations seen by Dubuisson and his colleagues (7-10). We agree with Jacob (10) that the fastest component in muscle extracts, his *h* and our Component D, is possibly the myoalbumin of Bate-Smith (2). Beyond this tentative identification we are not able to go at this time. Jacob has not been able to identify myosin. Neither has he identified myoglobin, which is clearly evident in our red muscle patterns. After some hours, our slow component, A, usually begins to split into two or three subfractions. The mobilities of this component and its fractions do not correspond with those of the slower elements of Jacob's patterns. Further correlation of our results with his is not at present possible.

## SUMMARY

1. An electrophoretic and ultracentrifugal study has been made of the proteins found in extracts of whole skeletal muscle, both red and white, and of heart muscle, taken from exsanguinated rabbits. The extracts have been secured in the 1st week after the application of various solutions to the tissues, which were frozen and thawed immediately after dissection. It is suggested that the procedure be called "mild extraction." The total protein extracted is about 1 per cent of the wet weight of the muscle.

2. Considerable amounts of the myogen complex have been removed within the 1st week by extraction with distilled water or with 0.4 M potassium phosphate buffer solutions at pH 7.6. Extraction proceeded at 0°. Several myogen components, A and D in white muscle extracts, and A, B (myoglobin), and D in red muscle extracts, have been detected in the elec-

trophoretic patterns and show characteristic mobilities, on the average nearly the same in both types of muscle. Components with similar mobilities have been observed in heart muscle.

3. The phosphate buffer solutions, which readily extract myosin from minced or homogenized tissue, did not remove it from carefully dissected whole skeletal muscles. A structural factor in the solubility of muscle proteins has thus been disclosed. In order to extract myosin, sodium pyrophosphate has been added in a concentration of 500 mg. per cent. This salt appears to break the link between actin and myosin in the fibrils so that the latter protein is free to diffuse.

4. The presence of myosin, in the extracts of red, white, and heart muscles, made with pyrophosphate, is demonstrated by (a) a considerable viscosity, increased by the addition of actin, and later diminished by the addition of adenosine triphosphate, (b) the appearance of a sharply defined elevation, Component C, in the electrophoretic patterns, and (c) the appearance of an equally sharply defined boundary in the ultracentrifuge sedimentation diagrams.

We wish to acknowledge, with great appreciation, the many courtesies shown to us in the Upsala laboratories by Dr. Arne Tiselius, Dr. The Svedberg, Dr. Kai O. Pedersen, and members of their staffs.

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# EFFICIENCY OF PHOSPHORYLATION COUPLED TO ELECTRON TRANSPORT BETWEEN DIHYDRODIPHOSPHOPYRIDINE NUCLEOTIDE AND OXYGEN\*

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Work in this laboratory has demonstrated that esterification of inorganic phosphate accompanies passage of electrons from dihydrodiphosphopyridine nucleotide (DPNH<sub>2</sub>) to oxygen in preparations of rat liver which consist largely of mitochondria (large granules) (1, 2). The esterification reaction was followed by measurement of the degree of incorporation of inorganic phosphate labeled with P<sup>32</sup> into the esterified phosphate fraction (easily hydrolyzable groups of adenosine triphosphate (ATP)). The isotopic technique was used because the enzyme preparations did not contain the transphosphorylases necessary to cause phosphorylation of secondary acceptors such as glucose, creatine, etc., by the ATP generated during the course of the oxidative phosphorylation. Measurements of this type, however, did not lend themselves readily to determination of the P:O ratio (moles of inorganic phosphate esterified to atoms of oxygen taken up) for reasons already discussed (1).

After the properties of the system had been more thoroughly established by the use of the tracer technique, it was found possible to measure the P:O ratio of this oxidation in non-isotopic experiments by using relatively high concentrations of adenosine diphosphate (ADP) or muscle adenylic acid (AMP) as phosphate acceptors. Under these circumstances disappearance of inorganic phosphate from the medium could be readily measured for calculation of P:O ratios. This paper deals with the P:O ratios observed during the DPN-linked oxidation of  $\beta$ -hydroxybutyrate to acetoacetate in the same type of enzyme preparation used in the previous study (2). Since the acetoacetate formed in this reaction does not undergo further oxidation, the observed phosphate exchanges may be attributed solely to electron transport between  $\beta$ -hydroxybutyrate and oxygen, and, further, since no phosphorylation has been observed during the interaction of substrate with DPN, it is probable that all the phosphate exchanges observed occur only on electron transport between DPNH<sub>2</sub> and oxygen (2).

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## EXPERIMENTAL

*Preparations*—The muscle adenylic acid used was prepared according to Kerr (3) and recrystallized from water several times. In addition the preparation marketed by the Ernst Bischoff Company, Inc. (My-B-den) was found to be entirely adequate for these studies. The ATP employed was a commercial preparation (Armour). The adenosine diphosphate (ADP) was prepared from ATP by an unpublished method utilizing the hexokinase reaction for removal of the third phosphate group. The preparation contained 13 per cent ATP but this type of impurity had no bearing on the usefulness of the preparation in this work.

*Methods*—Acetoacetate was determined colorimetrically as acetone by the method of Greenberg and Lester (4), following decarboxylation by incubation of aliquots of the trichloroacetic acid filtrates with aniline hydrochloride under properly standardized conditions. Inorganic and acid-labile P ( $\Delta 7P$ ) were determined by the standard Fiske-Subbarow method. The determination of inorganic pyrophosphate used was based on the method of Jones (5). Although the specificity and accuracy of the method were not extensively explored, it was found to give reasonably accurate recoveries and appeared to be completely specific for inorganic pyrophosphate in the trichloroacetic acid filtrates of the reaction media under study. An aliquot of a trichloroacetic acid filtrate containing at least  $1.0 \mu M$  of inorganic pyrophosphate was brought to pH 4.0 to 4.2 as nearly as possible with NaOH, with pH test paper. The volume was then made to 5.0 ml. with water. To this were added 5.0 ml. of 0.1 M acetate buffer, pH 4.1, 0.50 ml. of 10 per cent  $MnCl_2 \cdot 4H_2O$ , and 0.7 ml. of acetone, and the contents mixed. The pH was adjusted to 4.1 (glass electrode) if necessary. At this point a turbidity due to precipitation of manganous pyrophosphate was observed in tubes containing more than  $2 \mu M$ . Smaller amounts of pyrophosphate caused formation of turbidity on standing. The tubes were allowed to stand at room temperature overnight. They were then centrifuged and the supernatant discarded. The precipitated manganous pyrophosphate packed firmly and little difficulty was experienced due to losses of precipitate during decantation. The residue was washed with 2.5 ml. of 1 per cent  $MnCl_2 \cdot 4H_2O$  (prepared by dilution from the stock 10 per cent solution used above), recentrifuged, and the wash solution decanted and drained off as completely as possible. The residue was then dissolved in 0.1 N HCl and made to a convenient volume, an aliquot of which was used for determination of  $\Delta 7P$ . In the range from 1.0 to  $15 \mu M$  of pyrophosphate (2 to  $30 \mu M$  of  $\Delta 7P$ ) recovery from pure solution or from trichloroacetic acid filtrates of actual reaction media was excellent. At the lower level recovery was within 10 per cent; at the higher levels, within 2 to 3 per cent. Under these conditions,  $15 \mu M$  of ATP, 15

$\mu\text{M}$  of muscle adenylic acid, 8  $\mu\text{M}$  of DPN, or 28  $\mu\text{M}$  of inorganic orthophosphate added to trichloroacetic acid filtrates gave absolutely no turbidity nor did they cause interference in the recovery of known amounts of pyrophosphate. In this study the method has also been used in the separation of inorganic pyrophosphate from a mixture of ATP, ADP, inorganic orthophosphate, and DPN for determination of radioactivity in experiments in which inorganic orthophosphate labeled with  $\text{P}^{32}$  was used as a tracer. Inorganic pyrophosphate was separated from the filtrate as described above. It was then redissolved in 0.1 N HCl, brought to pH 4.1, and reprecipitated from this medium with  $\text{Mn}^{++}$  and washed. The precipitation was repeated a second time to yield a fraction essentially free of contaminating radioactive materials. Radioactivity measurements were performed as described before (2).

The enzyme preparations were made as described before (6); the washed residue was taken up in cold 0.15 M KCl. Suspensions of purified mitochondria (2, 7) were not used in the efficiency measurements, because it was feared that the lengthy procedure necessary might cause some inactivation of phosphorylating activity. Since maximum activity was required, the shorter procedure, although it yields preparations containing nuclei and erythrocytes as well as mitochondria, appeared to be most practical.

### Results

In a system composed of rat liver enzyme,  $\beta$ -hydroxybutyrate, DPN,  $\text{Mg}^{++}$ , cytochrome c, inorganic phosphate, either adenylic acid or adenosine diphosphate, and air as gas phase, inorganic phosphate disappears from the medium as the substrate is oxidized to acetoacetate and there is an accumulation of acid-labile P (7 minutes hydrolysis in 1 N  $\text{H}_2\text{SO}_4$  at  $100^\circ$ ) corresponding in amount to the inorganic phosphate lost. If 0.02 M sodium fluoride is present, the disappearance of inorganic phosphate is much greater. These basic findings are presented in Table I. Furthermore, it is clear from the data that in the absence of substrate there is no significant uptake of oxygen, and instead of a decrease of inorganic phosphate there is an increase due to hydrolysis of the adenine nucleotides. The data on oxygen uptake and acetoacetate formation agree very well, as in the previous study (2). The addition of DPN is absolutely necessary to demonstrate oxidation of  $\beta$ -hydroxybutyrate in this type of experiment. Although the fresh enzyme preparation contains a suboptimal amount of DPN, this appears to be completely destroyed by enzymatic action during the 5 minute equilibration at  $30^\circ$ .

The P:O ratios calculated from this type of experiment are about 1.0 in the presence of fluoride and about 0.4 to 0.6 in the absence of fluoride.

Since some liberation of inorganic phosphate from adenine nucleotide occurred even in the presence of fluoride, it appeared logical to expect that the true P:O ratio of the oxidation might be significantly greater than 1.0.

It was soon appreciated that another factor operated against determination of the maximum P:O ratio of the oxidation under these conditions.

TABLE I

*Net Removal of Inorganic Phosphate Coupled to Oxidation of  $\beta$ -Hydroxybutyrate to Acetoacetate*

The main compartment of the Warburg vessels contained 0.10 ml. of  $\text{MgCl}_2$  (0.005 M),\* 0.10 ml. of glycylglycine buffer, pH 7.45 (0.02 M), 0.10 ml. of cytochrome *c* ( $1.5 \times 10^{-5}$  M), 0.40 ml. of inorganic orthophosphate (0.005 M), 0.20 ml. of muscle adenylate (0.004 M) or ADP (0.003 M), 0.10 ml. of *dl*- $\beta$ -hydroxybutyrate (0.02 M) (or  $\text{H}_2\text{O}$ , as indicated), 0.10 ml. of DPN (0.0005 M), and 0.20 ml. of NaF (0.02 M) or water as indicated. The enzyme suspension, 0.50 ml., was placed in the side arm and tipped in after a 5 minute temperature equilibration period at 30°. The total volume was made to 2.00 ml. with  $\text{H}_2\text{O}$ . The reaction was stopped at zero time (end of equilibration period) or at 15 minutes by addition of trichloroacetic acid.

Flask No.	Substrate	P acceptor	NaF	Time	O <sub>2</sub> uptake	Acetoacetate formed	Inorganic P	$\Delta 7P$	$\frac{P}{O}$	P Acetoacetate
				min.	micro-atoms	$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$		
1	+	AMP	—	0		0.00	11.3	0.10		
2	+	"	—	15	3.56	3.40	9.55	3.82	0.49	0.51
3	—	"	—	15	0.18	0.10	13.6	0.10		
4	+	"	+	0		0.00	11.3	0.10		
5	+	"	+	15	3.36	3.40	7.55	3.82	1.08	1.10
6	—	"	+	15	0.13	0.10	12.5	0.00		
7	+	ADP	+	0		0.10	11.4	5.20		
8	+	"	+	15	3.28	3.15	8.29	8.22	0.94	1.02
9	—	"	+	15	0.18	0.10	12.9	3.10		

\* The figures in parentheses indicate the final concentration of each component in complete reaction medium. This notation is used in all tables.

Previous work had shown that incubation of the enzyme preparation by itself at 30° for short periods caused inactivation of the phosphorylation reaction without significant loss of oxidative activity (2). In the type of experiment described in Table I, the enzyme preparation was kept in the side arm for 5 minutes at 30° (the temperature equilibration period) before addition to the main compartment. It was found that this treatment caused great losses in phosphorylating ability. The temperature equilibration period is of course necessary for accurate manometric determination of oxygen taken up.

Since the amount of oxygen taken up is stoichiometrically related to acetoacetate formed during the oxidation (1 microatom of oxygen = 1  $\mu$ M of acetoacetate), it is possible to conduct measurements of the P:O ratio without manometric measurements of oxygen uptake simply by colorimetric determination of acetoacetate formed and inorganic phosphate taken up. In Table I it is seen that the ratio of P to acetoacetate is equivalent to the P:O ratio. Under these circumstances the complication of the equilibration period can be avoided, since rigid temperature control is not necessary. This approach has a second advantage in that the colorimetric determination of acetone by the modification of the method of Greenberg and Lester (4) is far more sensitive and accurate than manometric measurement of oxygen uptake when relatively small oxidative changes occur. As will be seen, the P:O ratio of the oxidation decreases with time. Obviously, measurement in the first few minutes will give more nearly maximum ratios. Over these short periods manometric measurements of oxygen uptake, even under the best conditions, are subject to relatively much larger errors than is the colorimetric estimation of acetoacetate.

By means of this technique measurements of the P:O ratios were carried out as follows: Ice-cold enzyme was added directly to the main compartment of the Warburg vessel with all necessary components already present and with trichloroacetic acid in the side arm. The vessel was immediately attached to the manometer and placed in the bath (25–30°). No manometric readings were taken. The oxidation was terminated by tipping the trichloroacetic acid into the main compartment. The filtrates were then analyzed for phosphate exchange and formation of acetoacetate.

With this approach it was possible to measure P:O ratios during reaction periods as short as 2 to 3 minutes. Although there was no rigid temperature control over at least the shorter reaction periods, the P:O ratio does not necessarily depend on temperature, although it is possible that the purely oxidative and the purely phosphorylative reactions may have somewhat different temperature coefficients.

With this approach a large number of determinations have been carried out. Each enzyme preparation was tested over several different time intervals. In Table II are shown the data collected from five consecutive enzyme preparations from a total of twelve tested. It is quite clear from the data that the P:O ratio of the oxidation measured under these conditions is significantly higher than the figure of 1.0 obtained by the type of experiment outlined in Table I. Also, it is obvious that phosphorylating ability declines with time, as has been observed in the tracer experiments. All of the ratios observed in the experiments listed in Table II are sufficiently greater than unity to allow the conclusion that probably at least

TABLE II

*Ratio of Inorganic Phosphate Esterified to Acetoacetate Formed during Oxidation of  $\beta$ -Hydroxybutyrate*

The vessels contained 0.10 ml. of  $MgCl_2$  (0.005 M), 0.10 ml. of glycylglycine buffer, pH 7.40 (0.02 M), 0.10 ml. of cytochrome *c* ( $1.5 \times 10^{-5}$  M), 0.10 ml. of *dl*- $\beta$ -hydroxybutyrate (0.02 M), 0.10 ml. of DPN (0.0003 M), 0.20 ml. of NaF (0.02 M), 0.40 ml. of inorganic phosphate, 0.40 ml. of adenylate (0.003 to 0.005 M). The ice-cold enzyme suspension, 0.50 ml., was added last to the otherwise complete medium (total volume, including enzyme, made up to 2.0 ml. with  $H_2O$ ) at timed intervals. Each vessel was immediately placed in a bath at 25° or 30°. The reaction was stopped by addition of 0.30 ml. of 20 per cent trichloroacetic acid from the side arm at the times stated.

Experiment No.	Time	Inorganic phosphate	Acetoacetate	P disappeared Acetoacetate formed
	<i>min.</i>	$\mu M$	$\mu M$	
1	0	11.17	0.00	
	4	8.45	1.26	2.16
	8	7.10	2.05	1.98
	9	7.23	2.23	1.77
	10	7.16	2.28	1.76
	11	6.45	2.72	1.77
	12	6.90	3.02	1.42
2	0	11.33	0.00	
	6	6.85	1.84	2.44
	9	5.55	2.67	2.16
	12	5.48	3.38	1.73
3	0	11.40	0.09	
	4	9.69	1.23	1.50
	8	8.78	2.10	1.30
	9	8.40	2.58	1.24
	10	8.00	2.58	1.36
	11	8.06	2.90	1.19
	14	7.10	2.40	1.30
4	0	11.20	0.00	
	8	7.32	1.76	2.20
	10	6.02	2.70	1.92
	12	4.90	3.35	1.88
5	0	12.40	0.00	
	4	9.31	1.39	2.22
	6	8.88	1.83	1.92
	7	8.10	2.22	1.94
	8	7.43	2.59	1.92
	8	7.31	2.71	1.88
	9	7.01	3.37	1.60
	12	6.80	3.42	1.64

2 molecules of inorganic phosphate are esterified for each pair of electrons passing from substrate to oxygen. In this series, five individual measure-

ments were greater than 2.0. Each of these represented measurements made near the beginning of the reaction, when the system would be expected to have maximum phosphorylating ability. Although the measurements of acetone and inorganic phosphate are least accurate at the beginning of the reaction for obvious reasons, the average error in the P:O ratio is probably no greater than  $\pm 10$  per cent. These five values derive more significance, however, when it is considered that none of the values

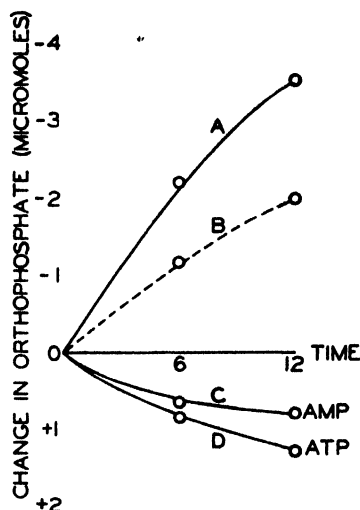


FIG. 1. Rate of phosphate uptake coupled to oxidation and rates of dephosphorylation of ATP and AMP. These experiments were arranged as in Table II and carried out at 30°. The concentration of AMP in both uptake and dephosphorylation experiments was 0.003 M and that of ATP in the dephosphorylation experiment was 0.002 M. Curve A represents the course of uptake of inorganic orthophosphate in the actively phosphorylating oxidation. Curve B represents the rate of formation of acetoacetate (also in micromoles) during the oxidation. Curve C represents the liberation of inorganic orthophosphate from AMP when the substrate for the oxidation ( $\beta$ -hydroxybutyrate) is omitted, and Curve D the liberation of orthophosphate from ATP ( $\beta$ -hydroxybutyrate omitted).

recorded has been corrected for the considerable dephosphorylation of adenine nucleotides which occurs simultaneously with the phosphorylation reaction.

In order to show the magnitude of such dephosphorylation losses experiments have been carried out in which the phosphate exchanges have been determined at two time intervals in a complete system showing aerobic phosphorylation. The same enzyme preparation was used to determine dephosphorylation losses during the same time intervals by omitting the substrate. The results of a typical experiment are shown in Fig. 1. It will be noted that both the phosphate acceptor (adenylic acid)

and the presumed phosphorylation product (ATP) are subject to dephosphorylation. The uncorrected P:O ratio of this particular experiment at 12 minutes was 1.76. If this figure is corrected upward by adding to the amount of inorganic phosphate found to disappear oxidatively ( $3.5 \mu\text{M}$ ) the amount of inorganic phosphate lost by dephosphorylation in the absence of substrate (the average of the loss from adenylic acid and ATP, or  $1.05 \mu\text{M}$ , will suffice for an approximation), then a total of approximately  $4.55 \mu\text{M}$  of inorganic phosphate is obtained. The P:O ratio, with this correction, becomes  $4.55/1.99$  or 2.28 in this particular experiment. Although this experiment did not show an uncorrected P:O ratio which was among the highest found, it does show the relative magnitude of the dephosphorylation loss and the approximate magnitude of the correction. The rates of dephosphorylation of ATP and AMP did not differ greatly among the different preparations tested. If the P:O ratios shown in Table II are corrected upward for such dephosphorylation losses, it would appear that a significant proportion of the total number of individual P:O ratios observed would be above 2.0.

Before the general validity of applying such corrections for dephosphorylation losses could be accepted, it was first necessary to examine two questions pertaining to interpretation of aerobic phosphorylation data.

The first question, regarding validity of dephosphorylation corrections, was brought up by Ogston and Smithies (8), who suggested that the measurements of ATPase activity in the presence of NaF, conducted by Ochoa (9) as part of his work on the efficiency of phosphorylation, may have been complicated by contamination of the ATP sample used with inorganic pyrophosphate. They suggested that true ATPase activity was actually completely inhibited by fluoride and that the observed formation of inorganic phosphate in the presence of fluoride arose not from ATP but from inorganic pyrophosphate presumed to be present as a contaminant of the ATP sample employed by Ochoa. They quote the observation of Lohmann (10) that the hydrolysis of inorganic pyrophosphate is not inhibited by fluoride in frog muscle as evidence that fluoride possibly did not inhibit inorganic pyrophosphate hydrolysis in Ochoa's experiments. The adenine nucleotides used in the present investigation were found to contain at the most only negligible quantities of inorganic pyrophosphate as determined by the rather sensitive and specific method already outlined. Such tests revealed that the samples of ATP used contained at the very most only 2 to 3 per cent of the  $\Delta 7\text{P}$  as inorganic pyrophosphate. Also, the effect of fluoride on the rate of dephosphorylation of adenylic acid, ATP, and inorganic pyrophosphate by the enzyme suspension used has been determined and is diagrammed in Fig. 2. It can be seen that

fluoride inhibited not only hydrolysis of ATP and adenylic acid but was also highly inhibitory to the hydrolysis of inorganic pyrophosphate. Inhibition of purified yeast pyrophosphatase by fluoride has been observed by Bailey and Webb (11). It would appear from these considerations that orthophosphate liberation from the adenine nucleotide samples used is not complicated by the presence of impurities of inorganic pyrophosphate and a selective inhibitory action of fluoride. For these reasons it is felt that these particular objections of Ogston and Smithies to correction of P:O ratios for dephosphorylation losses do not apply in this case.

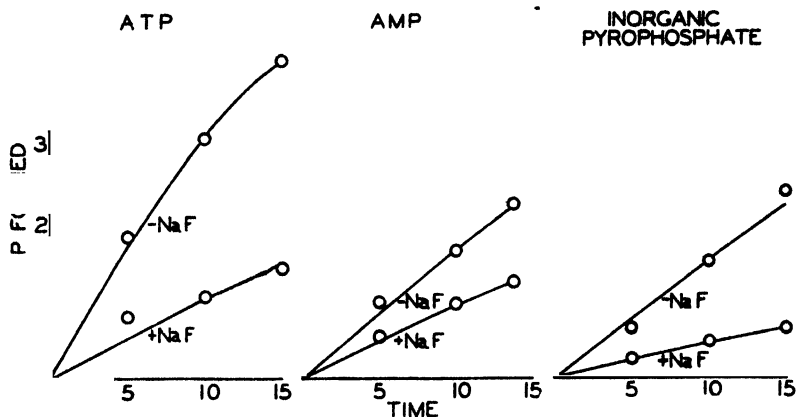


FIG. 2. Effect of 0.02 M NaF on rates of orthophosphate liberation from ATP, AMP, and inorganic pyrophosphate. The reaction medium and conditions were exactly the same as those described in Table II,  $\beta$ -hydroxybutyrate being omitted. Concentration of ATP, 0.002 M; AMP, 0.003 M; inorganic pyrophosphate, 0.002 M. Temperature, 30°.

There is a second point of uncertainty concerning the measurements of the P:O ratio and their interpretation. Cori and Ochoa (12) in unpublished work found that oxidation of glutamate in rat liver dispersions caused accumulation of inorganic pyrophosphate. More recently Cross *et al.* (13) have isolated inorganic pyrophosphate as a phosphorylation product after oxidation of Krebs' cycle intermediates by washed rabbit liver preparations similar to the rat liver preparations used in this study. The mechanism of origin of the inorganic pyrophosphate during oxidative phosphorylation has not been established. However, it now appears tempting to ascribe it to the occurrence of an enzymatic reaction recently discovered by Kornberg in yeast preparations (14). The enzyme described by Kornberg catalyzes the reversible reaction





Since the equilibrium constant of this reaction is about 0.4 according to Kornberg, the reaction could conceivably explain the appearance of inorganic pyrophosphate in the experiments quoted if this enzyme is present in animal tissues.

If this reaction does not occur in the preparations mentioned, the inorganic pyrophosphate may arise as an artifact, owing to decomposition of a "coenzyme pyrophosphate," as suggested by Ochoa (9) and Cross *et al.* (13), or it may actually arise as the product of the anhydridization of 2 molecules of orthophosphate coupled to the oxidation. In the latter case 2 molecules of orthophosphate would disappear for only one phosphate bond created, whereas if inorganic phosphate disappears by phosphorylation of adenylic acid, as has been generally assumed, disappearance of 1 molecule of orthophosphate is equivalent to the synthesis of one phosphate bond. If inorganic pyrophosphate is formed directly, the calculations of thermodynamic efficiency must necessarily take this into account. It therefore became of interest to determine whether the DPN-linked oxidation-phosphorylation under study caused the formation of inorganic pyrophosphate instead of, or in addition to, formation of adenosine polyphosphates.

By means of the method described for determination of inorganic pyrophosphate, trichloroacetic acid filtrates of media in which the oxidative phosphorylation described had taken place were examined for the presence of inorganic pyrophosphate. In no case could any accumulation of pyrophosphate be detected. Known amounts of inorganic pyrophosphate were readily recovered from such filtrates without any evidence of interference. In a typical large scale experiment it was found that 22.0  $\mu\text{M}$  of inorganic phosphate had disappeared, with the formation of 23.2  $\mu\text{M}$  of  $\Delta 7\text{P}$  (adenylic acid was the phosphate acceptor). No inorganic pyrophosphate could be detected in this filtrate, although an addition of 2.0  $\mu\text{M}$  of inorganic pyrophosphate to the filtrate could be recovered to the extent of 108 per cent. It would appear that if inorganic pyrophosphate were formed in this experiment the amount was considerably less than 10 per cent of the total  $\Delta 7\text{P}$  formed.

In order to determine more conclusively the rôle of inorganic pyrophosphate in the DPN-linked oxidation under study, experiments were performed in which the oxidative phosphorylation took place in the presence of inorganic orthophosphate labeled with  $\text{P}^{32}$  and unlabeled ATP and unlabeled inorganic pyrophosphate. Also present in the medium were the usual additions:  $\beta$ -hydroxybutyrate, DPN,  $\text{Mg}^{++}$ , cytochrome *c*, buffer, and sodium fluoride. After the reaction had taken place, the trichloroacetic acid filtrate was fractionated to determine to what extent the labeled inorganic phosphate had exchanged with the acid-labile P of ATP and

with the inorganic pyrophosphate. The total amount of the inorganic  $P^{32}$  incorporated into esterified form was determined by measurement of radioactivity remaining in solution after two magnesia precipitations of inorganic phosphate, each preceded by carrier dilution of inorganic  $P^{32}$  (2). The amount of  $P^{32}$  incorporated into the inorganic pyrophosphate was determined by separation of the inorganic pyrophosphate from another aliquot of the original trichloroacetic acid filtrate by precipitation with  $Mn^{++}$  at pH 4.1. The pyrophosphate salt obtained was redissolved in

TABLE III

*Non-Participation of Inorganic Pyrophosphate in Coupled Phosphorylation*

The reaction vessels contained 0.60 ml. of  $MgCl_2$  (0.005 M), 0.60 ml. of glycylglycine buffer, pH 7.35 (0.02 M), 0.60 ml. of cytochrome *c* ( $1.5 \times 10^{-5}$  M), 4.80 ml. of inorganic phosphate labeled with  $P^{32}$  (678,900 counts per minute), 4.80 ml. of ATP, 0.60 ml. of  $\beta$ -hydroxybutyrate (0.02 M), 0.60 ml. of DPN (0.0005 M), 2.40 ml. of NaF (0.02 M), 2.40 ml. of inorganic pyrophosphate, and 3.00 ml. of enzyme added directly to the medium. Total volume made up with  $H_2O$  to 12.0 ml. The reactions were fixed at zero time and after 12 minutes at 30° by addition of 1.80 ml. of 20 per cent trichloroacetic acid.

	Zero time	After incubation
Acetoacetate, $\mu M$ .....	0.00	16.4
Inorganic orthophosphate, $\mu M$ .....	55.2	54.2
Total $\Delta 7P$ , $\mu M$ .....	67.2	64.8
Inorganic pyrophosphate $\Delta 7P$ , $\mu M$ .....	44.4	39.4
ATP $\Delta 7P$ , by difference, $\mu M$ .....	22.8	25.4
Specific activity of inorganic orthophosphate, counts per min. per $\mu M P$ .....	12,300	8120
% $P^{32}$ in total esterified P .....	0.02	35.2
% " " inorganic pyrophosphate .....	0.29	1.08
Specific activity of inorganic pyrophosphate .....	44.3	186
% $P^{32}$ in ATP, by difference .....		30.6
Specific activity of $\Delta 7P$ of ATP, by difference .....		8170

acid and reprecipitated twice with  $Mn^{++}$ . The reprecipitated pyrophosphate, now essentially free of contaminating radioactive materials, was brought into solution in acid and the  $\Delta 7P$  and radioactivity determined.

The supernatant of the magnesia-treated aliquot used for determination of total esterified  $P^{32}$  was then brought to pH 7.0 and the ATP and inorganic pyrophosphate precipitated by addition of barium acetate. The precipitate was washed with 1 per cent barium acetate and then dissolved in 0.1 N HCl, and barium was removed as the sulfate by addition of a slight excess of  $Na_2SO_4$ . The filtrate was analyzed for  $\Delta 7P$  and for radioactivity.

The complete data of a typical experiment are presented in Table III.

It will be seen that acetoacetate was formed during the incubation due to oxidation of  $\beta$ -hydroxybutyrate. At zero time essentially no inorganic  $P^{32}$  was present in the Mg-soluble fraction or the inorganic pyrophosphate fraction, attesting to the completeness of separation of inorganic phosphate from the fractions. After the 12 minute oxidation period, however, a total of 35.2 per cent of the inorganic  $P^{32}$  had been converted into a form not precipitable by magnesia mixture and therefore presumably esterified. It will be noted that in this particular experiment there was no net uptake of inorganic orthophosphate, although considerable exchange had taken place. The inorganic pyrophosphate remaining at the end of the experiment, 88 per cent of that added at the beginning, contained only a total of 1.08 per cent of the  $P^{32}$ , probably not a significant amount of incorporation. The specific activity of this fraction was only some 2 per cent of that of the  $\Delta 7P$  of ATP. The Mg-soluble, Ba-insoluble fraction (largely ATP and ADP and inorganic pyrophosphate) contained 89 per cent of the esterified  $P^{32}$  of which 91 per cent was in the form of  $\Delta 7P$ , as already mentioned. Since the inorganic pyrophosphate fraction measured separately contained essentially no radioactivity, it may be presumed that whatever radioactivity was present in the "ATP" fraction must have been present in a form other than inorganic pyrophosphate. Since ATP (or probably a mixture of ATP and ADP) is the only major component of this fraction, it must be the component bearing the label. The data on specific activity of remaining inorganic orthophosphate and the  $\Delta 7P$  of the ATP show that the  $P^{32}$  was evenly distributed between the two fractions, or that complete equilibration had taken place.

Under the conditions employed in this experiment, then, inorganic phosphate does not undergo significant incorporation into inorganic pyrophosphate but does exchange readily with an esterified form of phosphate having the characteristics of the acid-labile groups of ATP. It may be concluded that inorganic pyrophosphate is not formed under these experimental conditions and that the reaction of Kornberg probably does not occur in this system. If it had, it would be expected that ATP bearing  $P^{32}$  (as a result of aerobic phosphorylation) would have come into equilibrium with inorganic pyrophosphate, since considerable DPN was present in this medium.

Although inorganic pyrophosphate formation has been observed in preparations of rat liver (12) and rabbit liver (13), it is possible that the enzymes necessary for its generation have been separated from the particulate material used in this study by the washing procedure employed in preparation of the suspension or that the system was inactive under the test conditions used.

The former explanation was borne out by other experiments, the results

of which may be mentioned briefly. A comparison was made between the well washed particulate material used in these experiments and a crude, unfractionated homogenate of rat liver with respect to the ability of each type of preparation to cause incorporation of inorganic orthophosphate into inorganic pyrophosphate. Conditions were exactly as described in Table III. Although the specific activity of inorganic pyrophosphate in experiments with well washed enzyme never exceeded about 2 per cent of that of the  $\Delta 7P$  of ATP, when crude homogenates were used there was extensive incorporation into pyrophosphate approaching that found in the ATP  $\Delta 7P$ , indicating that whole rat liver homogenate does have the ability to form pyrophosphate. However, other experiments revealed that the rate of incorporation of inorganic phosphate into inorganic pyrophosphate in whole rat liver homogenate is considerably lower than the rate of incorporation into ATP, suggesting that pyrophosphate formation is not on the main pathway of aerobic phosphorylation.

These experiments allow the tentative conclusion that the phosphorylation process studied involves as the over-all reaction the conversion of adenylic acid to ADP or ATP by phosphorylation coupled to the oxidation without the participation of inorganic pyrophosphate. For this reason it is felt that the P:O ratios may be legitimately corrected for dephosphorylation losses due to hydrolysis of the adenine nucleotides without complications arising from a possible participation of inorganic pyrophosphate.

Although a correction may appear legitimate, its accurate mathematical expression is not simple to derive owing to the dynamic changes in concentrations of components with time. In any event a significantly large proportion of the individual measurements, if corrected by what seem to be reasonable factors, falls above 2, and since the coupled esterification appears to be a quantized phenomenon, then a value of 3 might be postulated for this oxidation, as has been done for other oxidations (9, 13).

#### DISCUSSION

The P:O ratios observed for the isolated oxidation reaction studied are quite comparable to values obtained for Krebs' cycle oxidations by Belitzer and Tsibakova (15), Ochoa (9), and more recently by Cross *et al.* (13). Each oxidative step of the Krebs cycle, except the oxidation of succinate to fumarate, appears to cause the esterification of somewhat more than 2 molecules of orthophosphate. If some correction upward is allowed for dephosphorylation losses, the P:O ratio may be as high as 3.0.

If the normal electrode potentials of the dehydrogenase-substrate system ( $-0.293$  volt at pH 7.0 (16)) and of the oxygen electrode ( $+0.81$  volt at pH 7.0 and 0.2 atmosphere of  $O_2$  partial pressure) are used for calculation of the maximum free energy change on passage of a pair of

electrons from substrate to oxygen, then  $\Delta F = -nFE = -2 \times 23,068 \times (0.29 + 0.81) = -50,700$  calories. Since each labile P bond of ATP requires at least 12,000 calories for generation, then a maximum of  $50,700/12,000 = 4.23$  bonds can be formed. The data presented here indicate an observed yield of two bonds or a measured efficiency of some 47 per cent. If correction of the basic data for dephosphorylation losses is permissible, the efficiency may be correspondingly higher. For reasons already considered (2) it appears probable that all of the observed phosphorylation in the system studied occurs on passage of electrons from DPNH<sub>2</sub> to oxygen. Since the normal electrode potential of the pyridine nucleotide system is nearly identical with that of the  $\beta$ -hydroxybutyrate-acetoacetate system ( $-0.28$  volt (17) compared to  $-0.293$  volt (16)), the calculations of efficiency already given on the basis of the  $\beta$ -hydroxybutyrate system as electron donor apply also to the case in which the DPNH<sub>2</sub> is regarded as electron donor with only a small error. However, it must be emphasized that the P:O ratios studied have been observed with the  $\beta$ -hydroxybutyrate system and not with DPNH<sub>2</sub> as substrate. Some experiments have been carried out with DPNH<sub>2</sub> in high concentration as substrate in an effort to determine the P:O ratios directly. However, several factors weigh against demonstration of maximum ratios under these conditions. Manometric measurement of oxygen uptake is necessary in this case and not only is subject to the difficulties of enzyme inactivation already described but also becomes rather unwieldy owing to the very high rate of oxidation of DPNH<sub>2</sub> by these preparations. In addition, it has already been reported (2) that high concentrations of nucleotide are inhibitory to phosphorylation. Theoretically it is possible for enough energy to be liberated during the interaction of  $\beta$ -hydroxybutyrate with DPN to cause some phosphorylation, but only near the beginning of the oxidation, when the ratio of  $\beta$ -hydroxybutyrate to acetoacetate is very high and the ratio of DPNH<sub>2</sub> to DPN is quite low. However, as has been mentioned before (2), attempts to demonstrate phosphorylation during this phase of the over-all oxidation process have met with failure. Also, judging from the general pattern of DPN-linked dehydrogenase systems, it appears unlikely from both the mechanistic and energetic view-points to expect a phosphorylation to occur during interaction of  $\beta$ -hydroxybutyrate with DPN.

More rigidly controlled experiments to establish the validity, magnitude, and mathematical form of correction for dephosphorylation losses have not been attempted. It appears more profitable at this stage in our knowledge of oxidative phosphorylation to devote research efforts to elucidation of basic mechanisms rather than to refinement of efficiency measurements.

## SUMMARY

The ratio of moles of inorganic phosphate esterified to atoms of oxygen taken up during the course of the DPN-linked oxidation of  $\beta$ -hydroxybutyrate to acetoacetate in washed rat liver preparations has been studied. P:O ratios approaching 2.0 were consistently observed. All the phosphorylation which occurred can probably be attributed to the oxidation of DPNH<sub>2</sub> by molecular oxygen. It appears possible that this value should be corrected upward, owing to the fact that both adenylic acid, the phosphate acceptor, and ATP, the presumed phosphorylation product, undergo dephosphorylation losses, the magnitude of which has been indicated. Tracer experiments with P<sup>32</sup> showed that inorganic pyrophosphate is not formed as a phosphorylation product during the course of the oxidation in the liver preparations studied.

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# PARTIAL PURIFICATION AND PROPERTIES OF A PROTEOLYTIC ENZYME OF HUMAN SERUM\*

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That mammalian blood contains a proteolytic enzyme was first reported by Dastre (1). Reviews of the subsequent literature pertaining to the proteolytic enzyme have been published recently by Christensen and MacLeod (2), by Kaplan (3), and by Rocha e Silva and Rimington (4).

The inactive form of the proteolytic enzyme of animal serum has been named, by various investigators, "serum tryptogen" (5), "profibrinolysin" (6), and "plasminogen" (2), while the active form has been called "serum tryptase," "fibrinolysin," and "plasmin." Unfortunately, the term "fibrinolysin" was originally used, and is still used by bacteriologists, to denote a factor, elaborated by streptococcal bacteria, which activates the serum protease. In this report, we shall use the terms "plasminogen" and "plasmin" to refer to the two forms of the enzyme, while "streptokinase" will refer to the bacterial activator. The term "proenzyme" will be used synonymously with "plasminogen."

The principal objects of this study were to discover methods for purifying plasminogen and to elucidate further the properties of both the active and inactive forms of the enzyme. Partial purification of the enzyme has been reported previously by Edsall, Ferry, and Armstrong (7), by Loomis, George, and Ryder (6), and by Rocha e Silva and Rimington (4). We have succeeded in obtaining purification of plasminogen by a factor of 136- to 165-fold compared to the original serum. We present the following results to provide information to others who may wish to attempt further purification of plasminogen.

## *Materials and Methods*

*Serum*—Except where otherwise stated, the serum used in these studies was prepared from dried human plasma supplied by the American Red Cross. The dried plasma was reconstituted in distilled water plus sufficient dilute acetic acid to lower the pH to 7.8 to 8.0. Following solution of the proteins, the fibrinogen was clotted at 35° by additions of human

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or bovine thrombin concentrates. The fibrin clots were collapsed by stirring and removed shortly after their formation. A final centrifugation at approximately  $1500 \times g$  was used to remove the small shreds of fibrin.

*Streptokinase*—Concentrates of streptokinase were prepared by the method of Christensen (8), by use of a human group C strain of *Streptococcus hemolyticus* (H46A) kindly supplied to us by Dr. Christensen.

*Casein Solutions*—Commercial preparations of casein were found unsuitable for assay purposes because of their high content of acid-soluble nitrogen. The casein used in this study was precipitated from milk at pH 4.7. It was dissolved in water plus dilute NaOH and reprecipitated two or more times by addition of dilute HCl. The precipitated protein was washed successively with water, ethanol (95 per cent), and ethyl ether, and then dried *in vacuo*. For use the casein was dissolved in a solution of 0.1 M phosphate, 0.9 per cent NaCl, and sufficient NaOH to adjust the solution to pH 7.40. The solution was immersed in boiling water for 15 minutes, filtered, and the pH readjusted to 7.40. After a preliminary nitrogen determination, the solution was diluted to the desired concentration, usually 5 gm. of casein per 100 ml. of solution.

*Hippuramide*—This compound was prepared and purified by the method of Fischer (9). The product melted at  $186^{\circ}$  (reported,  $183^{\circ}$ ). A micro-Kjeldahl determination showed 15.40 per cent nitrogen (theory, 15.73). The product gave a negative Nessler's test, which became slightly positive in 30 minutes and strongly positive on further standing.

*Phosphate-Saline Buffer*—Unless otherwise stated, the plasminogen preparations, streptokinase concentrates, and casein used in enzyme assays were dissolved in 0.1 M potassium phosphate-0.9 per cent NaCl solution previously adjusted to pH 7.40. When necessary the resulting solutions were adjusted to pH 7.40, either by dialysis against an excess of the same buffer or by addition of dilute NaOH or dilute acetic acid.

*Determination of Acid-Soluble "Tyrosine"*—For this determination the method of Heidelberger and MacPherson (10), with minor modifications, was used. The reducing capacity of each test solution was expressed in terms of the equivalent amount of pure tyrosine.

*Determination of Proteolytic Activity*—An appropriate amount of plasminogen solution was placed in a 25 ml. Erlenmeyer flask, and diluted to 0.8 ml. with phosphate-saline buffer. 0.3 ml. of 5 per cent casein solution in phosphate-saline buffer and a predetermined excess of streptokinase concentrate, contained in 0.2 ml., were added. The mixture was incubated at  $35^{\circ}$  for 10 minutes to permit complete activation of the plasminogen. 3.7 ml. of 5 per cent casein were then added, and the combination mixed thoroughly. 2 minutes from the time of adding casein, an aliquot (2 ml.) was withdrawn and added to an equal volume of 10

per cent trichloroacetic acid. Incubation of the remaining sample was continued at 35° for 1 hour, when a second aliquot was withdrawn and treated in the same manner. After 20 to 30 minutes the precipitated protein was removed by filtration through Schleicher and Schüll No. 576 paper. An appropriate aliquot (usually 1 ml.) of each filtrate was transferred to a colorimeter tube, diluted to 2 ml. with 5 per cent trichloroacetic acid, and the acid-soluble tyrosine determined by the procedure cited above and by reference to the standard tyrosine curve. Units of acid-soluble tyrosine per complete digest were calculated from the results for the 2 and 62 minute aliquots, the increase being taken as a measure of the proteolytic activity.

*Proteolytic Unit*—1 proteolytic unit was arbitrarily taken as that amount of enzyme producing an increase of 450  $\gamma$  of acid-soluble tyrosine in a medium of 4 per cent casein in 1 hour.

*Nitrogen*—The total nitrogen content of impure samples of plasminogen was determined by the micro-Kjeldahl procedure. Samples of lower nitrogen content were analyzed by digestion and nesslerization.

## EXPERIMENTAL

### *Preliminary Studies*

*Demonstration of Proteolysis*—Two preliminary experiments were made for the purpose of demonstrating true proteolysis by human plasma or a fraction thereof. In Experiment 1, 10 ml. of whole plasma were incubated at 37° with 20 ml. of phosphate-saline buffer and 20 ml. of a streptokinase preparation. The amount of streptokinase used was adequate to cause lysis of a fibrin clot formed from the same quantity of plasma in 4 to 6 minutes. At the same time a control was run in which phosphate-saline buffer was substituted for streptokinase. In Experiment 2, samples of "lysin factor" (11, 2), prepared from fresh human serum and from the serum of dried and reconstituted plasma, were used as plasminogen sources, while casein was added as the substrate. 1 ml. of each lysin factor solution was incubated with 1 ml. of streptokinase concentrate for 10 minutes. 12 ml. of 5 per cent casein solution were added to each flask and incubation continued at 37°. The acid-soluble tyrosine and nitrogen were determined, in both Experiments 1 and 2, after incubation for the periods indicated in Table I.

The results for Experiments 1 and 2 are presented in Table I. It is apparent that in Experiment 1, in which whole plasma was used, very little proteolysis could be detected. Though there was lysis of the fibrinogen in the test sample (addition of thrombin produced no clot), the active enzyme was apparently neutralized quickly by the serum inhibitor (2). In Experiment 2 appreciable proteolysis occurred, as is shown by

the large increase in both acid-soluble tyrosine and nitrogen. These experiments demonstrated the necessity of fractionating an original serum before its content of plasminogen can be determined. Usually the original activity of a given serum was determined by the preparation and assay of a lysin factor sample, a recovery of 100 per cent of the enzyme being assumed. This assumption seems warranted because of the extremely low solubility of the enzyme, under these conditions, when purified fractions are tested.

*Determination of Optimal Substrate Concentration*—Direct proportionality between enzyme concentration and the effect measured requires that the enzyme be saturated with respect to substrate. Since this pro-

TABLE I  
*Proteolysis by Plasmin before and after Removal from Whole Plasma*

Experiment No.	Sample	Incubation time	Acid-soluble tyrosine	Acid-soluble nitrogen
			$\gamma$ per ml. digest	mg. per ml. digest
1	Whole plasma, test	1 min.	15.1	0.054
		25 "	19.6	0.058
		192 "	21.8	0.059
	" " control	1 "	14.0	0.042
		192 "	13.0	0.044
2	Lysin factor from dried plasma	1 "	59	0.10
		90 "	440	0.84
		13 hrs.	991	1.94
	Lysin factor from fresh serum	1 min.	54	0.11
		90 "	452	0.84
		13 hrs.	775	1.85

portionality is desirable for quantitative assay of the enzyme content of various fractions, an experiment was conducted to determine the substrate (casein) concentration necessary for saturation of the enzyme. Constant amounts of plasminogen and streptokinase were incubated 30 minutes at 35° to insure complete activation. At the end of this time, casein of constant volume but varying concentration was added, the casein having been diluted with phosphate-saline buffer. The increase in acid-soluble tyrosine during 1 hour's incubation at 35° was then determined in the usual manner. The first experiment on optimal substrate concentration was made with casein which had been precipitated from fresh skim milk and reprecipitated twice. The total volume of digesting medium was 7.5 ml. The results, shown graphically in Curve 1, Fig. 1, were surprising. Concentrations of casein above 1.0 per cent caused diminishing amounts of tyrosine to be converted to the acid-

soluble condition. Since it appeared possible that these results depended upon the presence of inhibitors in the casein preparation, the experiment was rerun with casein which had been reprecipitated a total of six times after removal from skim milk. The results, shown in Curve 2, Fig. 1, confirmed those of the first trial, in so far as the two experiments were comparable. The actual magnitudes of the effects in the two experiments, for a given casein concentration, do not coincide, because two different enzyme preparations were used. The final concentration of enzyme in the first experiment was 1.02 proteolytic units per digest; that in the second, 1.25 proteolytic units. Suboptimal amounts of sub-

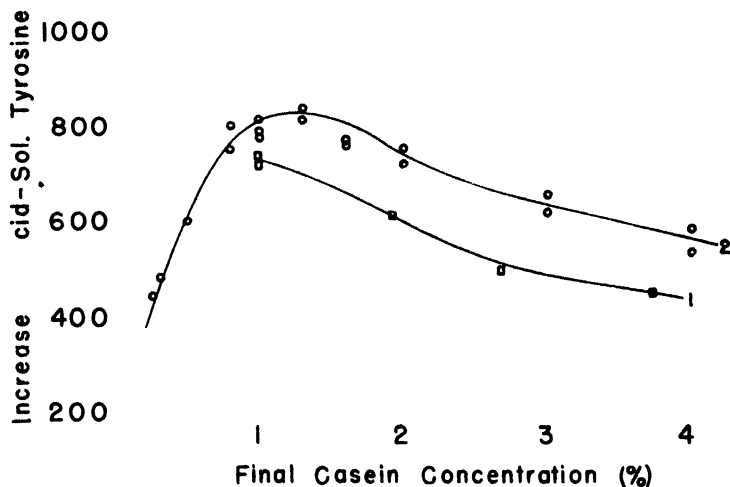


FIG. 1. Curves for casein substrate concentration. The increases in acid-soluble tyrosine are in terms of micrograms per 7.5 ml. of digestion medium. Curve 1 was obtained with casein reprecipitated two times; Curve 2, with casein reprecipitated six times.

strate are indicated below the 1.0 per cent casein level. As the substrate concentration was increased above 1.4 per cent casein, diminishing amounts of tyrosine were converted to the acid-soluble condition.

*Relation between Time and Effect Measured*—Since the curves for substrate concentration indicated possible inhibition by the accumulation of end-products, time curves were run at two different concentrations of casein. Four flasks were set up, each containing 11.3 units of proenzyme and an excess of streptokinase, the total volume in each flask being 13.5 ml. The flasks were incubated 30 minutes at 35°. 54 ml. of casein were then added to each flask. In two flasks, 5 per cent casein was added, giving a final concentration of 4 per cent. In the other two flasks, 1.6 per cent casein was added, giving a final concentration of 1.28 per cent.

The flasks were then incubated at 35°, aliquots being withdrawn periodically for determination of acid-soluble tyrosine and nitrogen as described previously. The results, calculated on the basis of 7.5 ml. digests, are shown in Fig. 2. Both acid-soluble tyrosine and nitrogen continued to increase for 4 hours in the medium containing 4 per cent casein. After an initially slower rate, the acid-soluble material increased almost linearly, beginning to drop off during the 4th hour. In the medium of 1.28 per cent casein, the rate of conversion was higher during the 1st hour, but began to drop off after digestion for 80 to 100 minutes.

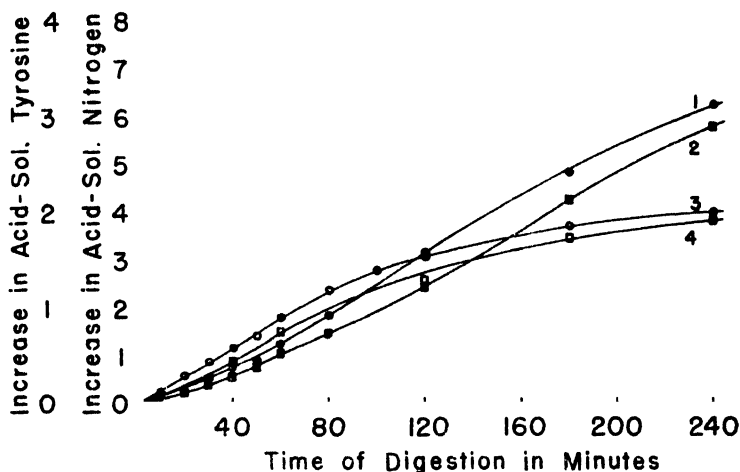


FIG. 2. Time curves for digestion of casein by plasmin. Values for tyrosine and nitrogen are in terms of mg. per 7.5 ml. of digestion medium. Curve 1, production of acid-soluble tyrosine in 4 per cent casein; Curve 2, production of acid-soluble nitrogen in 4 per cent casein. Curves 3 and 4 correspond respectively to Curves 1 and 2, but were obtained in a medium of 1.28 per cent casein.

A final casein concentration of 4 per cent was chosen for routine assays. Lower levels of substrate offered the possibility of a greater magnitude of the effect measured (increase in acid-soluble tyrosine). However, it was considered probable that the 4 per cent level would give better proportionality between the units of enzyme employed and the effect measured. Furthermore, the range in which this proportionality would apply could be expected to be larger at the higher level.

*Proportionality between Enzyme Concentration and Effect Measured*—The validity of assuming strict proportionality between enzyme concentration and effect was tested directly. A final concentration of 4 per cent casein was used, since this concentration had been chosen for routine assays. Varying amounts of plasminogen (0.28 to 2.80 units) were

activated with an excess of streptokinase by incubating at 35° for 30 minutes, the final volume in each case being 1.5 ml. 6 ml. of 5 per cent casein were then added to each flask. The increase in acid-soluble tyrosine during 1 hour was then determined in each case. The results are shown in Table II. The error between duplicate flasks was 8.5 per cent at the lowest concentration. However, this concentration was never used in determining the enzyme content of critical samples. If the approximate degree of purification warranted reinvestigation, dilute samples were concentrated by  $(\text{NH}_4)_2\text{SO}_4$  precipitation or by isoelectric pre-

TABLE II  
*Relation between Enzyme Concentration and Production of Acid-Soluble Tyrosine*

Enzyme per flask		Increase of acid-soluble tyrosine	Apparent P. U. per ml.*
ml.	total P. U.	$\gamma$ per digest	
0.1	0.28	121	2.69
0.1	0.28	111	2.47
0.2	0.56	238	2.65
0.2	0.56	238	2.65
0.4	1.12	508	2.82
0.4	1.12	498	2.76
0.6	1.68	760	2.81
0.6	1.68	735	2.72
0.8	2.24	1008	2.80
0.8	2.24	1008	2.80
1.0	2.80	1170	2.60
1.0	2.80	1173	2.61
0.0	0.00	0	0.00

\* Apparent units of plasminogen per ml. of enzyme solution added

cipitation at low ionic strength. The error between duplicates at concentrations from 0.56 to 2.8 units was 0 to 3.25 per cent. The departure from strict proportionality between 0.56 and 1.68 units, the range used routinely, was 5.1 per cent.

### *Purification Experiments*

It was suspected at the beginning of this study that once the serum protease became active it would prove to be unstable. Particularly in the presence of inhibitors would this be true, as indicated by the work of Christensen and MacLeod (2) and by our preliminary studies. No attempts were made, therefore, to isolate the activated enzyme. In fact spontaneous activation, encountered occasionally, has generally made fur-

ther purification from the point of its inception an almost hopeless task. Purification of the inactive enzyme is preferable from another standpoint; namely, that the mechanism of activation could then be studied more satisfactorily. In the purification experiments to be reported, the enzymatic activity per mg. of nitrogen (P. U. per mg. of N) was used as the sole criterion for the degree of purification attained.

*Early Purification Results*—Soon after the beginning of the present study, we accomplished purification of plasminogen by a factor of approximately 335-fold, calculated from the original serum. The procedure used for this purification included alcohol fractionation, precipitation by  $\text{Na}_2\text{SO}_4$  at pH 5.4, adsorption upon and elution from kaolin, fractionation in dilute trichloroacetic acid at pH 3.75, and a second adsorption upon and elution from kaolin. The over-all recovery of active material, in the purest fraction, was quite low (1.9 per cent), while most of the enzyme could be accounted for in impure fractions. Subsequent experiments revealed that such a procedure was not reliable, and, further, that plasminogen samples showed inconsistent behavior. In particular, part of the enzyme precursor centrifuged upward when precipitated with  $\text{Na}_2\text{SO}_4$ , while part of it centrifuged downward. Plasminogen showed widely variable solubility in the trichloroacetic acid fractionation, and this appeared to depend upon the previous history of the material used. Some fractions were found to give very poor results when purification was attempted with kaolin as an adsorbent. Such results made it appear inadvisable to continue with the same methods of fractionation.

*Alcohol Fractionation*—In view of the greater number of variables offered by water-ethanol mixtures (12), it was hoped that such systems would enable a critical separation of the proteolytic enzyme of human serum. A large number of alcohol fractionations were performed under the following conditions: While alcohol was being added, and during the subsequent period of stirring, the temperature of the water-ethanol system for each fractionation was held at the freezing point or not more than  $2^\circ$  above this temperature. A 49.2 volume per cent solution of ethanol was used for establishing the alcohol concentrations. In water systems, with no ethanol present, the temperature was routinely held at  $2-5^\circ$ . The precipitates were removed from suspension by quick centrifugation (3 to 5 minutes) at approximately 5000 R.P.M. in a model SS-1 Servall, angle head centrifuge. This speed of centrifugation was chosen because of the apparently contradictory results, and to allay doubts as to whether precipitated proteins were being removed completely. During the high speed centrifugations, the temperature of the water-ethanol systems occasionally rose from  $0^\circ$  to  $+2^\circ$ .

The results of extensive efforts to purify plasminogen with water-

ethanol mixtures may be summarized as follows: Immediately after precipitation from serum, either by ammonium sulfate or ethanol (at pH 7.0 or above), the proenzyme appears to possess a very low solubility under the following conditions: pH 7.0 to 8.0, ionic strength from 0 to 0.05, ethanol concentrations of 10 to 15 volumes per cent. Under these conditions, considerable total protein precipitates from the primary fractions of serum. Upon further purification, especially by precipitation at 0.08 ionic strength and pH 5.3, the proenzyme exhibits a much higher solubility under the conditions stated previously, and at the same time very little, if any, protein is precipitated. Changes in apparent solubility by factors of several fold have been encountered frequently under supposedly fixed conditions but at different stages of purification. We have, in one experiment, reversed this change of solubility by combining the fractions separated in the isoelectric precipitation.

The pronounced tendency of plasminogen to precipitate with other proteins has, in general, resulted in distribution of the proenzyme into every fraction separated, too frequently in proportions similar to those in which total protein was distributed. Some increases in purity have been obtained, but always with the loss of a large share of the enzyme in impure fractions.

*Precipitation by Ammonium Sulfate*—Precipitation by ammonium sulfate has not proved to be an effective method for purifying plasminogen in this laboratory. Our results indicate that a solution of partially purified plasminogen (from human plasma) must be adjusted to approximately 0.4 saturation with ammonium sulfate to cause essentially quantitative precipitation, while part of the active material is precipitated at 0.28 saturation. As the concentration of ammonium sulfate is increased in the range between 0.28 and 0.4 saturation, there appears to be no critical point of precipitation of plasminogen. Attempts to fractionate with this reagent have usually resulted in dispersal of plasminogen into several fractions, with only minor degrees of purification. We have employed ammonium sulfate principally as a reagent for concentrating dilute solutions of plasminogen.

*Isoelectric Precipitation*—The experiments to be described here were suggested by Christensen's report (8) that the proteolytic enzyme could be removed from human serum by dialysis or dilution to low ionic strength, followed by acidification to pH 5.3. That this precipitate could be purified further at pH 5.3 and an ionic strength of 0.08 was indicated by the work of Edsall, Ferry, and Armstrong (7). Freshly prepared serum was dialyzed with stirring against several changes of distilled water, for periods of 10 to 57 hours. Dialysis for the longer periods was applied when a large volume of serum was involved. This treatment would be expected



to give an ionic strength less than 0.001. The dialyzed serum was then adjusted to pH 5.3, and the precipitated proteins removed by centrifugation at 5000 R.P.M. in the Servall angle head centrifuge. By this means, a purification of 7- to 10-fold was accomplished. The precipitate was then subjected to isoelectric precipitation from 0.08 M NaCl as shown in the purification diagram. The results of five experiments (A to E), in which this procedure was used, are shown in Table III. The over-all recovery varied between 61 and 72 per cent. Fractionation by successive isoelectric precipitations at the two ionic strengths has proved not only

TABLE III  
*Purification of Plasminogen by Isoelectric Precipitation*

Experiment No.	Fraction	Original purity	Enzyme concentration*	Ionic strength†	Purity of ppt.
		<i>P. U. per mg. N</i>	<i>P. U. per ml.</i>		<i>P. U. per mg. N</i>
A-1	Serum, dialyzed 10 hrs.	0.14	1.36	<0.001	1.08
A-2	Ppt. of A-1	1.08	0.68	0.08	4.73
B-1	Same as in A-1	0.14	0.63	<0.001	1.22
B-2	Ppt. of B-1	1.22	0.80	0.08	4.68
C-1	Serum, dialyzed 57 hrs.			<0.001	
C-2	Ppt. of C-1		2.13	0.08	4.00
D-1	Serum, dialyzed 14 hrs.	0.12	1.09	<0.001	1.10
D-2	Ppt. of D-1	1.10	0.70	0.08	4.54
E-1	Serum, dialyzed 50 hrs.	0.12	1.11	<0.001	
E-2	Ppt. of E-1		0.60	0.08	5.21

\* Enzyme (plasminogen) concentrations are those existing immediately prior to centrifugation.

† The values, <0.001, are estimates indicating the approximate salt concentration after dialysis of the serum, with stirring, against several changes of distilled water for the time indicated.

to be a reliable approach to the problem of purification, but it has also resulted in plasminogen samples which give favorable results when purified further by adsorption upon kaolin.

**Kaolin Adsorption**—As mentioned previously, the plasminogen of certain fractions could be purified further by adsorption on, and elution from, kaolin. This method of purification had been abandoned for a considerable period during this investigation, because of the large losses encountered in its use. However, subsequent experiments with other methods have revealed the probability that heavy losses may be unavoidable. Fractionation by adsorption on kaolin not only offered the highest degree of purification, but it appeared to be a more reliable procedure for purification beyond 50- to 80-fold. Thus it seemed advisable to verify the

results of kaolin adsorption with several fractions previously prepared by a standard procedure.

*Partial Purification of Plasminogen*

Serum, dialyzed to low ionic strength

Adjust to pH 5.3 with dilute acetic acid; centrifuge

Ppt.

Dissolve in 0.16 M NaCl, pH 7.0-7.4; dilute to 0.08 M NaCl and approximately 1 proteolytic unit per ml.; adjust to pH 5.3 with dilute acetic acid; centrifuge

Supernatant  
(discard)

Ppt.

Dissolve in phosphate-saline buffer, pH 7.40; centrifuge to remove insoluble proteins

Supernatant  
(discard)

Supernatant

Dialyze against 0.3 M NaCl; dilute to 1-2 proteolytic units per ml. by addition of 0.3 M NaCl; adjust to pH 5.0 by addition of dilute acetic acid or an acetate buffer; add 1 gm. kaolin per 80-100 proteolytic units; stir 1½-2 hrs.; centrifuge

Ppt.  
(discard)

Kaolin

Suspend with stirring 15-20 min. in each of two portions of 0.1 M phosphate-0.9% NaCl buffer, pH 5.4; centrifuge after each period of stirring

Supernatant  
(discard)

Kaolin

Suspend with stirring 3-4 hrs. in phosphate-saline buffer, pH 7.40, total volume half that during adsorption step; centrifuge

Two washings  
(discard)

Eluate

Contains plasminogen, 15 to 19 proteolytic units per mg. N

Kaolin  
(discard)

The fractions chosen for this study were the precipitates formed at ionic strength 0.08 and pH 5.3, as indicated in Table III. The procedure

for the adsorption experiments is described in the last three steps of the flow chart of the entire purification process. The specific data and the results of seven adsorption experiments are indicated in Table IV.

Analysis of the kaolin, after adsorption and elution of plasminogen, indicates that some protein and plasminogen remain on the kaolin. A second elution with the phosphate-saline buffer removes negligible amounts of plasminogen which was relatively impure. Attempts to remove all of the plasminogen, by use of phosphate buffers under varying conditions, have proved unsuccessful.

TABLE IV  
*Purification of Plasminogen by Adsorption on Kaolin\**

Experiment	Enzyme employed	Volume	Kaolin added	Enzyme in 1st eluate	
					Purity
	<i>P. U.</i>	<i>ml</i>	<i>gm.</i>	<i>per cent†</i>	<i>P. U. per mg. N</i>
A	26.9	23	0.30	29.9	19.0
B	26.9	23	0.30	30.2	18.9
C	26.9	30	0.30	29.8	19.1
D	98.6	44	1.10	25.8	15.8
E	49.3	30	0.55	25.8	16.3
F	49.3	40	0.55	23.3	17.7
G	295.8	220	3.30	24.4	17.2

\* In Experiments A to C, the original material was a fraction of proteins precipitated at ionic strength 0.08 and pH 5.3, as indicated in Table III. This fraction possessed 4.54 P. U. per mg. of N. In Experiments D to G, the original material was a similar fraction possessing 5.21 P. U. per mg. of N. The volume indicates the total ml. of suspension during the adsorption phase

† Per cent of the total enzyme committed to adsorption.

### *Kinetic Studies*

*Kinetics of Activation*—In view of Ratnoff's report (13) that plasminogen is activated by a stoichiometric reaction with streptokinase, at least under certain conditions, it was considered of interest to study this reaction. Two proenzyme preparations were used, the first one proving unsuitable because of the presence of inhibitors. This first preparation was a sample of lysin factor, containing 1.85 proteolytic units per ml. Evidence for the presence of inhibitors in this sample was later discovered in that the sample showed a negative dilution effect on assaying at two concentrations. The other sample had been purified further by isoelectric precipitation at 0.08 ionic strength, and showed no dilution effect. It contained 2.46 proteolytic units per ml. The procedure was as follows: 5 ml. of the proenzyme were mixed and incubated with 0.2 ml. of streptokinase at 35°. Aliquots (0.7 ml.) were withdrawn periodically, added to

casein solutions, and assayed for active enzyme by the usual 1 hour's incubation. The logarithm of the ratio of the total activity attainable ( $C_1$ ) to the amount of enzyme ( $C_2$ ) not activated at the end of each time interval was calculated, and these values were plotted against the time of incubation in minutes. The results of the first series are shown in Curve 1, Fig. 3. An excessive concentration of streptokinase had been used, making the results valueless. However, it was noted that the activity dropped considerably after the initial, rapid activation. The

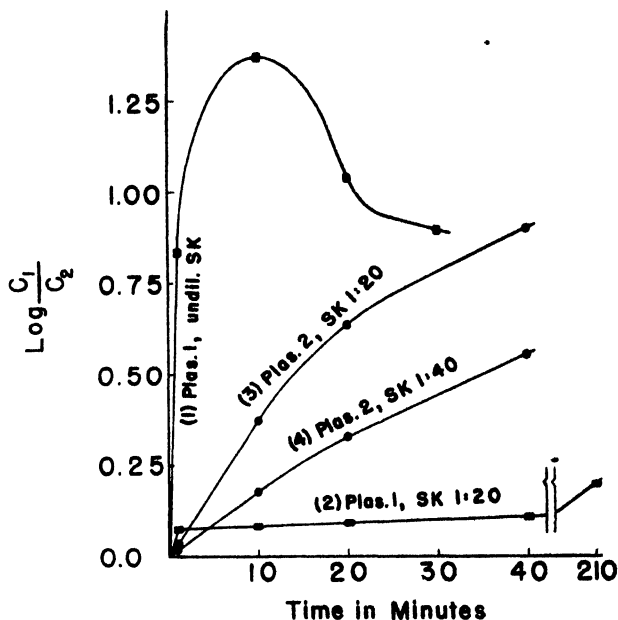


FIG. 3. Rates of activation of plasminogen by streptokinase. Plasminogen Sample 1 (Curves 1 and 2) contained 1.85 proteolytic units per ml.; Sample 2 (Curves 3 and 4) contained 2.46 proteolytic units per ml. Streptokinase (SK) was diluted as indicated. Explanation of  $\log C_1/C_2$  presented in the text.

procedure was then repeated with the same materials, except that the streptokinase had been diluted 1:20. The results are shown in Curve 2, Fig. 3, and they indicate again the presence of inhibitors. It should be mentioned, however, that activation did continue even after 40 minutes. The results on the first sample made inadvisable any further study with it as the enzyme source. The procedure described above was then repeated with the second proenzyme sample and streptokinase at 1:20 and 1:40 dilutions. The results are shown in Curves 3 and 4, Fig. 3.

A first order reaction is indicated with the second sample, since the slope of Curve 3 is approximately double the slope of Curve 4 during the

first 10 minutes. However, the results after the first 10 minutes are complicated by obvious destruction of part of the enzyme.

*pH of Optimal Activity*—Since two recent reports (14, 15) indicate that more than one proteolytic enzyme may be present in human serum, two different plasminogen preparations were used in this study. One of these was a preparation of lysin factor, having 1.08 proteolytic units per mg. of nitrogen, while the other had been further purified by adsorption on, and elution from, kaolin. The latter preparation had 15.8 proteolytic units per mg. of nitrogen. The two enzyme preparations were diluted to approximately the same concentration, according to their assays with casein as the substrate. In these experiments a final concentration of 0.8 per cent casein was used because a preliminary run at 4 per cent casein showed a low response to change in pH. 0.8 ml. of the two enzyme preparations was placed in two series of flasks. Activation was accomplished by addition of 0.2 ml. of streptokinase. 5 minutes incubation at 35° were allowed for activation, and this was done at pH 7.40 in all cases. 4 ml. of casein (1 per cent in M/15 phosphate buffer) were then added to each flask, the pH being variable. Incubation was continued at 35°, aliquots being withdrawn at 2 and 52 minutes after addition of casein. The increase in acid-soluble tyrosine was then determined by the procedure described previously. The results are shown graphically in Fig. 4. It should be mentioned here that at values above pH 7.7 the pH dropped during the incubation. In these instances the averages of initial and final pH values were plotted, while at all lower pH levels only the initial pH was plotted. It may be seen that both enzyme preparations showed optimal activity between pH 7.30 and 7.50.

*Fibrinolysis*—To test further the hypothesis that human serum may contain more than one proteolytic enzyme, we have compared the relative activities of two plasmin preparations against two substrates. No synthetic substrates for plasmin have been reported. However, it was felt that the use of two proteins might give a similar type of evidence. The relative activities of plasmin samples against (1) casein and (2) fibrin clots were compared. One of the plasminogen samples was a preparation of lysin factor containing 1.08 P. U. per mg. of N; the other consisted of material purified to 19.0 P. U. per mg. of N by adsorption on kaolin. The two samples were diluted so that 0.8 proteolytic unit was contained in each ml. of the two separate preparations. The dilutions were made in accordance with the assay values when production of acid-soluble tyrosine from casein was measured. The two preparations were then compared for the rates of fibrin lysis. The procedure for determining lysis time was as follows: The plasminogen, contained in a volume of 0.5 ml., was activated for 3 minutes at 35° with an excess (0.1 ml.) of strepto-

kinase. At zero time, 0.3 ml. of a bovine fibrinogen solution (0.33 per cent clottable) was blown into the tube, followed immediately by 0.1 ml. of bovine thrombin. The tubes were incubated at 35° and the times of lysis noted. This latter time extended from the moment that fibrinogen was blown into the tube until lysis was complete. All of the materials added were dissolved in 0.9 per cent NaCl at pH 7.4, except that the

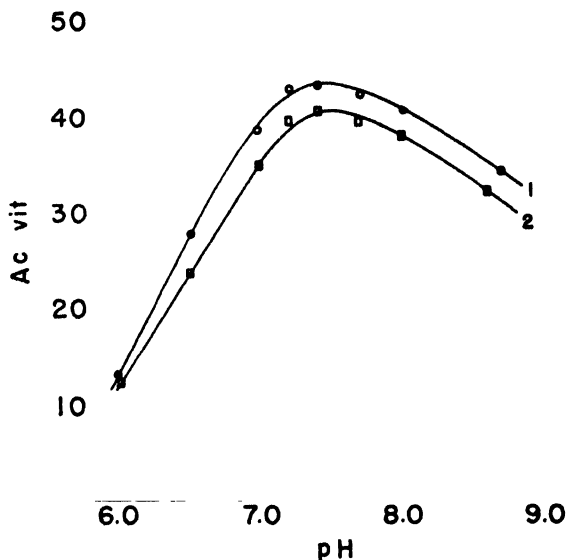


FIG. 4. pH of optimal activity for two preparations of plasmin. Activity is expressed in terms of the increase in micrograms of acid-soluble tyrosine per ml. of the digestion medium. Curve 1 was obtained with a sample of lysin factor with 1.08 proteolytic units per mg. of N; Curve 2, with a kaolin eluate containing 15.8 proteolytic units per mg. of N.

fibrinogen solution contained, in addition, 0.01 M potassium phosphate buffer and an unknown concentration of sodium citrate.

In the interest of brevity the results of such comparisons, at five different concentrations of plasmin, are summarized. Except at the lower concentration of enzyme, lysis of the fibrin clots followed the kinetics of a first order reaction. That is, the product of plasminogen or plasmin concentration and the lysis time was constant for all but the lowest enzyme concentration. At the lowest concentration, lysis occurred more rapidly than was expected. When plasminogen from the two samples described above was added to establish the same concentration, according to their

assays with the casein substrate, the lysis times were the same, within the limits of experimental error. This indicated that the two enzyme preparations, differing in purity by a factor of 17.6-fold, possessed the same ratio of activities toward the two substrates, casein and fibrin.

*Attempted Hydrolysis of Hippuramide*—Fruton (16) reported that rabbit serum contains an endopeptidase which hydrolyzes hippuramide. It occurred to us that hippuramide might serve as a substrate for plasmin. Different plasminogen preparations were activated by streptokinase and used in three separate experiments designed to detect the hydrolysis of hippuramide. Our results were completely negative, since in no instance did the formol titration of test mixtures exceed that of control mixtures. We conclude that the plasmin of human serum does not hydrolyze hippuramide.

#### DISCUSSION

The occurrence, in animal serum, of inhibitors of plasmin has long been known. Though we have made no study of such inhibitors, our preliminary experiments demonstrate the necessity of removing the enzyme from serum before appreciable proteolysis can be detected.

Attempts to purify plasminogen have indicated a property of the proenzyme which, to our knowledge, has not been reported previously. Our results are consistent with the hypothesis that plasminogen possesses a marked facility for coprecipitation with, or adsorption upon, other precipitating proteins. Under widely varying conditions, in water-ethanol systems, in dilute trichloroacetic acid, and in aqueous solutions at pH values above 7.0, plasminogen was found to precipitate whenever appreciable protein was precipitated. Under similar conditions, but after previous removal of a large share of the contaminating proteins, plasminogen exhibits a much higher solubility. Though inexplicable losses of the enzyme were encountered occasionally, the total enzyme used in a given fractionation procedure could generally be accounted for by assay of all the resulting fractions. The apparent change of solubility of plasminogen upon purification could not, therefore, be explained simply on the basis of enzyme denaturation.

Successive isoelectric precipitation, first at very low ionic strength and then at 0.08, proved to be the most effective approach to the problem of purifying plasminogen. For purification beyond approximately 50- to 80-fold, adsorption upon, and elution from, kaolin gave the best results. Though purifications by factors of 136- to 165-fold were obtained, such preparations appear to contain considerable protein other than plasminogen. In our opinion, more highly specific methods, which circumvent the

possibility of coprecipitation, must be found before plasminogen can be purified completely.

Contradictory results have been obtained concerning the mechanism by which streptokinase activates plasminogen. Ratnoff (13) reported a stoichiometric relationship for this reaction when accomplished under certain circumstances. Christensen and MacLeod (2) had reported a first order, catalytic mechanism for the activation process. Our results confirm those of Christensen and MacLeod (2) when a plasminogen preparation, believed to be low in inhibitor content, was employed. When a preparation of plasminogen was used which apparently contained inhibitors, inconclusive results were obtained. We suggest that the stoichiometric reaction described by Ratnoff (13) may depend upon a reaction between streptokinase and the antistreptokinase reported by Kaplan (17).

Recent reports (14, 15) suggest that two proteolytic enzymes may be present in human serum. Our results do not exclude this possibility, but they do indicate that only one proteolytic enzyme is appreciably active after incubation of preparations of human plasminogen with streptokinase.

The authors wish to express their appreciation to Dr. L. R. Christensen for providing a pure strain of *Streptococcus hemolyticus*, and for his many helpful suggestions during the early stages of this work.

#### SUMMARY

1. Plasminogen was purified by 136- to 165-fold compared to the original human serum.
2. Inconstant behavior of plasminogen samples during fractionation procedures, suggesting coprecipitation with other proteins, made further purification difficult with these techniques.
3. Some evidence is given for the presence in human serum of only one proteolytic enzyme activated by streptokinase.

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# THE PARTIAL PURIFICATION AND PROPERTIES OF ANIMAL AND PLANT HYDANTOINASES

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Hydantoin is rapidly hydrolyzed to hydantoic acid by suspensions of the livers of omnivorous animals (1). Kidney suspensions hydrolyze it more slowly, but other organs are not active. The enzyme responsible for the hydrolysis is absent from all the organs of Herbivora such as the rabbit and guinea pig. It is present, however, in jack bean meal, in beans, peas, and their hulls, and in cucumber and melon seeds. The mammalian and jack bean enzymes show identical specificities in that neither di-substituted hydantoins nor parabanic acid is hydrolyzed (2). They differ, however, in certain properties such as Michaelis constant, pH optimum, effects of temperature, and sensitivity to various drugs. The following is a description of the partial purification of the two enzymes and some quantitative data on their activity.

## EXPERIMENTAL

The enzyme activity was measured manometrically in a solution containing 0.05 M Na-K-phosphate and 0.05 M  $\text{NaHCO}_3$  in equilibrium with a gas mixture of 5 per cent  $\text{CO}_2$  and 95 per cent nitrogen. The hydantoic acid formed displaced  $\text{CO}_2$ , but the end-point of the reaction was less than the theoretical. When the evolution of  $\text{CO}_2$  ceased, aliquots of the solution were analyzed for hydantoic acid by the method of Archibald (3) and compared with ammonium hydantoinate made according to the method of Lippich (4), which had been incubated with the enzyme under exactly the same conditions as the hydantoin. The results showed that when  $\text{CO}_2$  evolution ceased all the hydantoin was converted to hydantoic acid. It was thus possible to use the manometric method for the quantitative estimations. The colorimetric method was also used for certain experiments. The pH optima of the liver and jack bean enzymes were determined in this way in borate buffer, since accurate pH determinations of solutions equilibrated with  $\text{CO}_2$  are difficult. The optimum for the liver enzyme is pH 8.2, for the jack bean pH 8.6. The manometric determinations were all done at pH 7.8 in phosphate buffer.

The liver enzyme was prepared from rat liver which was ground in a mortar with 25 ml. of 0.05 M Na-K-phosphate buffer of pH 7.8. The

insoluble material was removed by centrifugation and discarded. 20 per cent acetic acid was added to the supernatant drop by drop until no further precipitation occurred. Usually 10 drops were sufficient. The precipitate was centrifuged and the supernatant dialyzed overnight in the ice box. It was then warmed to 40°, and this produced a precipitate which was increased by the addition to 2 to 3 drops of 1 per cent  $\text{NaHCO}_3$ . This precipitate was again centrifuged and discarded. 3.0 ml. of 95 per cent alcohol were added to every 10 ml. of the supernatant. The precipitate was centrifuged and discarded and the supernatant evaporated to dryness at room temperature under a fan. It was then taken up in 5.0 ml. of  $\text{H}_2\text{O}$  and centrifuged once more. The clear solution, containing some hemoglobin, had high enzymatic activity.

The jack bean enzyme was prepared from the meal supplied by The Arlington Chemical Company. To 5.0 gm. of meal, 25 ml. of borate buffer, pH 8.5, were added and the mixture allowed to stand for 4 hours at room temperature. The material was centrifuged and the supernatant dialyzed overnight in the ice box. The turbid solution was evaporated to half its volume under a fan at room temperature and centrifuged again. It remained turbid and was used as such. Both enzymes retained their activity for at least a week in the ice box. The liver preparation contained small amounts of various esterases but no oxidative enzymes. The jack bean preparation contained urease and allantoinase. Urease has no effect on either hydantoin or allantoin, but it was possible that allantoinase was responsible for the hydrolysis of both, even though the liver enzyme has no effect on allantoin. Three experiments show that different enzymes in jack bean meal are responsible for the hydrolysis of these compounds. In the first, undialyzed extract of the meal was precipitated by various concentrations of alcohol and the ratio of allantoinase to hydantoinase activity determined in each precipitate. Equimolar concentrations of the substrates were used and the relative rates of hydrolysis determined during a 2 hour period. In the untreated extract the ratio of allantoinase to hydantoinase activity was 0.50. In the precipitate formed by 30 per cent alcohol the ratio was 0.15, in the 50 per cent alcohol precipitate it was 0.41, and in the 80 per cent alcohol precipitate, 0.57. Thus hydantoinase is precipitated at lower alcohol concentrations than allantoinase, and the two enzymes can be virtually separated by 30 per cent alcohol. Secondly, pea hulls contain large amounts of hydantoinase but little allantoinase, the ratio of the two being 10:1. Bean hulls, however, contain more allantoinase than hydantoinase in a ratio of 7:5. Finally, dimethylhydantoin was added to the dialyzed jack bean meal extract and its effect on the hydrolysis of equimolar concentrations of hydantoin and allantoin measured. At a concentration of

0.05 M, dimethylhydantoin inhibits the hydrolysis of hydantoin 65 per cent but has no effect on the hydrolysis of allantoin.

Both enzymes are inactivated by acetone but not by alcohol. Both are stable in alkaline solutions but unstable in acid, and in this respect the liver enzyme is more sensitive than the jack bean. As shown below, cyanide inhibits the liver enzyme at much lower concentrations than the jack bean. The differential is greater than is indicated by the figures because the liver enzyme solution contains methemoglobin which binds some of the added cyanide. The relative sensitivity of the two enzymes to sodium arsenite is the same as to cyanide. When two solutions of equal activity are compared,  $3.0 \times 10^{-3}$  M arsenite inhibits the hydrolysis by the liver 80 per cent, by the jack bean 11 per cent. Twice this concentration inhibits the latter only 18 per cent. This differential is not due to extraneous sulfhydryl groups in the jack bean preparation, for if it is heated in a boiling water bath for 2 minutes to inactivate the enzyme and then added to the liver preparation, the latter is not protected against the action of the arsenite. As shown below, metrazol inhibits both enzymes equally. In high concentrations, parabanic acid and procaine inhibit; glycine and urea in concentrations of 5.0 mg. per ml. have no effect.  $1 \times 10^{-2}$  M iodoacetate inhibits the jack bean enzyme 30 per cent, the liver 20 per cent.

*Rate of Reaction*—In nearly all experiments it was found that the rate could be represented very accurately by the first order equation,  $kt = \ln a/(a - x)$  (Fig. 1), where  $a$  is the initial substrate concentration; the exceptions were a few experiments in which wide but irregular deviations occurred, and these were rejected. Not only did the rate in the early part of the experiment follow this equation, but there was no divergence even when 90 per cent or more of the substrate was hydrolyzed, as shown in Fig. 1, where the dotted line indicates the amount of  $\text{CO}_2$  evolved on complete hydrolysis. The relationship of velocity to substrate concentration given by the Michaelis-Menten equation is

$$\frac{dx}{dt} = \frac{V_{\max.} (a - x)}{(a - x) + K_m} \quad (1)$$

When  $(a - x)$  is small with respect to  $K_m$ , this reduces to the first order equation,  $dx/dt = k(a - x)$ . Since in our experiments  $K_m$  was found to be 0.016 and 0.032 respectively for the two enzymes, as shown below, and  $a$  usually varied between 0.005 and 0.05, this condition was not fulfilled. It would therefore be expected that the velocity equation could be derived by integrating equation (1) as it stands; this leads to the equation recently derived in a different way by Van Slyke (5).

$$V_{\max} t = x + K_m \ln \frac{a}{(a - x)} \quad (2)$$

Our data, however, fit the first order equation instead of this. Although it is not probable, it is still possible that the divergence might be ascribed to the products of the reaction causing a progressive decrease in the rate, exactly balancing  $x$ , the first term of the right hand side of equation (2), and thus reducing the expression to the first order equation. That this is not so is shown by an experiment in which 10.0 mg. of hydantoic acid

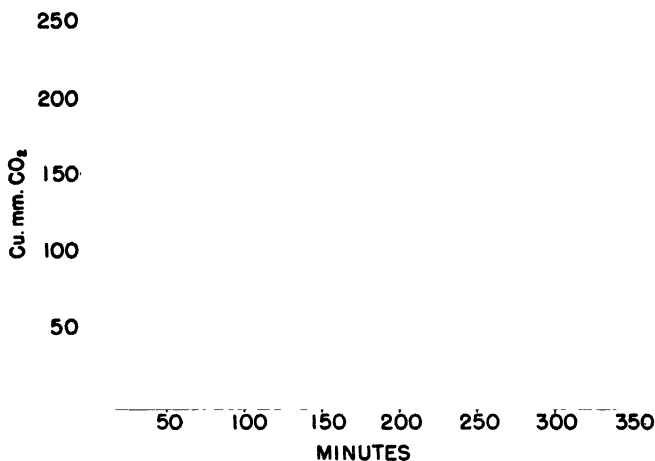


FIG. 1. Hydrolysis of 2.0 mg. of hydantoin in the presence of liver enzyme at 37.5°. The ordinate represents c.mm. of CO<sub>2</sub> evolved. The dotted line gives the amount evolved when complete hydrolysis has occurred.

failed to affect the rate of hydrolysis of 2.0 mg. of hydantoin by either the liver or jack bean enzyme.

For each experiment  $\log (a - x)$  was plotted against  $t$ , and the equation of the "best" straight line determined by the method of least squares. The slope thus obtained and corrected for the difference between common and natural logarithms is the velocity constant,  $k$ .

*Michaelis Constant*—The velocity constant,  $k$ , was plotted against the initial velocity, obtained by multiplying the velocity constant by the initial substrate concentration,  $a$ . As a rule the points fell fairly close to the straight line predicted by the Michaelis-Menten equation when written in the form

$$v = V_{\max} - K_m \frac{v}{a} \quad (3)$$

An example is given in Fig. 2. No evidence was observed of any tendency towards a systematic deviation from such a line. The slope of

this line is the Michaelis constant, and it was computed from the data by least square methods. Results of consecutive experiments were pooled

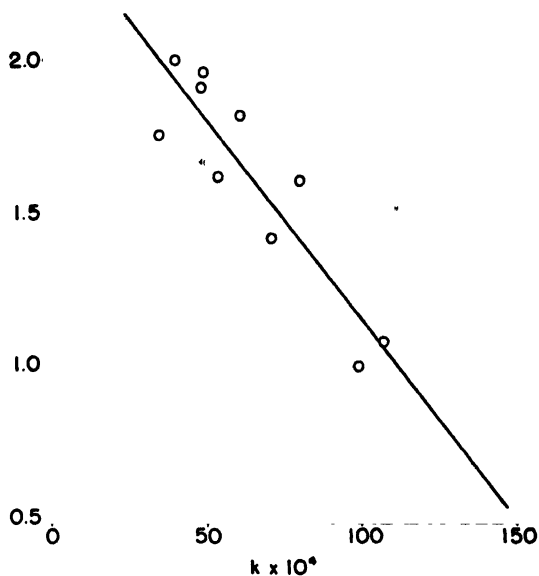


FIG. 2. The Michaelis constant at  $37.5^\circ$  for jack bean enzyme. The ordinate gives initial velocity of hydrolysis (moles per minute  $\times 10^4$ ); the abscissa gives the velocity constant ( $k \times 10^4$ ). The slope of the line (0.013) is the Michaelis constant for this experiment.

TABLE I

*Michaelis Constant for Liver and Jack Bean Enzymes at  $37.5^\circ$*

Liver		Jack bean	
No. of separate rate determinations	Pooled values of $K$	No. of separate rate determinations	Pooled values of $K$
5	0.0122	5	0.0143
11	0.059	15	0.0136
22	0.029	19	0.0167
26	0.031	22	0.0161
30	0.031	25	0.0170
34	0.029	30	0.0156
40	0.031	35	0.0155
46	0.032	41	0.0158
		44	0.0159
		48	0.0159

until an approximately stable value was obtained. Table I shows the values obtained by successive pooling for each of the enzymes for experi-

ments at 37.5°. It is evident that further experiments are unlikely to alter these values materially. The *F* test showed that the individual values of the constant do not vary more than might be expected from the errors involved in their separate determinations. It will be seen that the constant for the liver enzyme is almost exactly twice that for the jack bean enzyme which, therefore, has a greater affinity for the substrate.

*Effect of Temperature*—With use of a colorimetric method and a substrate concentration of 0.02 M, the velocity constant, *k*, was determined at four different temperatures between 0° and 37.5°. In Fig. 3 the reciprocal

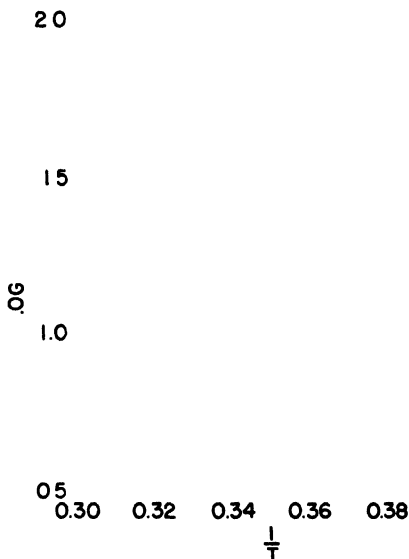


FIG. 3. The effect of temperature on the rate of hydrolysis of hydantoin by liver enzyme. The ordinate gives the velocity constant ( $k \times 10^4$ ); the abscissa gives the reciprocal of the absolute temperature.

of the absolute temperature is plotted against  $\log k$  for one of these experiments. It is clear that the points lie very close to the straight line of the Arrhenius equation,

$$\log k = \log A - \frac{E}{R} \times \frac{1}{T}$$

From the slope of this line the activation energy (*E*) may be calculated. As with the determination of the Michaelis constant, the results of successive experiments were pooled until a relatively stable value was obtained. Table II shows the successive values obtained in this way for each enzyme. It is apparent that the energy of activation for the reac-

tion as catalyzed by the jack bean enzyme is approximately twice that found in the case of the liver enzyme.

The effect of changes of temperature on the Michaelis constant was also investigated. By the manometric method, the Michaelis constants for each enzyme were determined at 24°, and at 2°, and compared with those already found at 37.5°. Table III shows from pooled data of suc-

TABLE II  
*Activation Energy for Liver and Jack Bean Enzymes*

No. of separate rate determinations	<i>E</i> from pooled data	
	Liver	Jack bean
	<i>calories</i>	<i>calories</i>
2	6765	12,908
4	6145	11,445
6	5662	11,874
8	5476	12,171
10	5274	11,903
12	5335	11,627

TABLE III  
*Effect of Temperature on Michaelis Constant*

Liver, 2°		Liver, 24°		Jack bean, 2°		Jack bean, 24°	
<i>N</i>	<i>K<sub>m</sub></i>	<i>N</i>	<i>K<sub>m</sub></i>	<i>N</i>	<i>K<sub>m</sub></i>	<i>N</i>	<i>K<sub>m</sub></i>
6	0.0250	6	0.0442	5	0.0219	6	0.0146
12	0.0398	12	0.0310	11	0.0167	12	0.0162
18	0.0390	18	0.0333	17	0.0149	18	0.0165
24	0.0372	24	0.0337	23	0.0145	24	0.0167
30	0.0345			28	0.0139	30	0.0175
				34	0.0158	36	0.0176
				40	0.0158		

*N* = total number of separate rate determinations.

cessive experiments that the effect of change of temperature on the Michaelis constant is practically nil for each enzyme. This means that the heat of formation of the enzyme-substrate compound is zero in each case. This is in agreement with results of Nelson and Blomfield (6) (but see discussion by Stearn (7)).

It is tempting to identify the enzyme-substrate compound with the "activated" molecule of Glasstone, Laidler, and Eyring (8). If we do so, we may calculate the following values in calories:



	$\Delta H$	$\Delta F$	$\Delta S$
Liver . . . . .	0	+1838	-6.68
Jack bean . . . . .	0	+2264	-8.23

However, the proof of identity of these two theoretical substances is lacking, and it is entirely possible that activation occurs as a further stage after the formation of the enzyme-substrate compound, as believed, for instance, by Kaufman, Neurath, and Schwert (9). These figures must therefore be taken with caution (see also Lineweaver and Burk (10)).

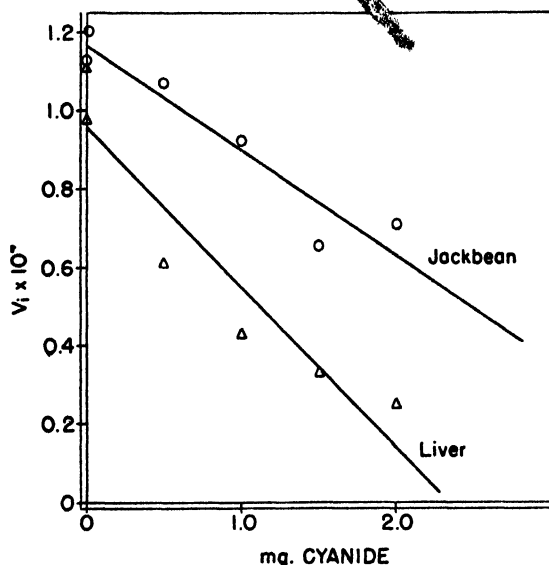


FIG. 4. The effect of cyanide (mg. in 2.0 ml.) on the rate of hydrolysis of hydantoin by liver and jack bean enzymes at 37.5°. The ordinate gives the initial velocity (moles per minute  $\times 10^4$ ); the abscissa gives the mg. of cyanide added.

*Inhibition*—Differences between the two enzymes are also shown by the effect of cyanide on the rate of hydrolysis (Fig. 4). Each mg. of cyanide added to the jack bean enzyme in the presence of 0.04 M hydantoin decreases the velocity constant by 0.27. In the case of the liver enzyme under similar conditions the decrease is 0.41,  $1\frac{1}{2}$  times as great.

Both metrazol and dimethylhydantoin act as competitive inhibitors; on plotting the velocity constants,  $k$ , against initial velocity, as in the determination of the Michaelis constant given above, it was found that the slope of the line had changed; theoretically the slope is now  $K_m(1 + i/K_i)$  instead of  $K_m$ . Table IV shows the results of pooling successive

values for the slope for each enzyme and for the two inhibitors. In all cases  $F$  tests were not significant, indicating that the values obtained in individual experiments did not vary more than expected when the errors of separate determination were considered. Values for  $K_i$  were then computed from the final figures in each column and are as follows:

	Metrazol	Dimethylhydantoin
Liver . . . . .	0.013	0.125
Jack bean . . . . .	0.013	0.044

The affinity of each enzyme for metrazol appears to be the same, and is in each case greater than that for hydantoin. The affinity of the two enzymes for dimethylhydantoin is different, but in neither case greater than that for hydantoin.

TABLE IV

*Inhibition by Dimethylhydantoin and Metrazol of Liver and Jack Bean Enzymes*

The figures represent the pooled values for the slope  $K_m (1 + i/K_i)$ .

Liver				Jack bean			
Dimethylhydantoin		Metrazol		Dimethylhydantoin		Metrazol	
<i>N</i>		<i>N</i>		<i>N</i>		<i>N</i>	
4	0.041	4	0.077	5	0.050	4	0.033
8	0.033	10	0.058	11	0.025	7	0.034
14	0.033	16	0.050	17	0.030*	11	0.021
20	0.039	22	0.050	23	0.030	17	0.027
26	0.042					23	0.025

*N* = total number of separate rate determinations.

#### DISCUSSION

At one time hydantoins were considered possible intermediates in the metabolism of amino acids. No evidence for the presence of such compounds has been found in analyses of tissues and urine. Lazarev (11), however, claims that incubation of glycine and ammonia with liver slices yields hydantoic acid. We have been unable as yet to confirm this, but the possibility remains that some glycine may be metabolized to hydantoin. It is the only amino acid which would form unsubstituted hydantoin and the enzyme is apparently specific for this compound. In plants also glycine may form hydantoin, for many contain an extremely active hydantoinase. Extracts from pea hulls, for instance, have been obtained which hydrolyze 1 mg. of hydantoin per minute. Purification of this enzyme will yield a tool with which it may be possible to analyze animal and plant tissues for the presence of hydantoin.

## SUMMARY

The partial purification of hydantoinases in animal and plant tissues has been described. The rate of hydrolysis catalyzed by these enzymes follows the first order equation. The enzymes obtained from liver and jack bean respectively have properties differing as follows: optimum pH. 8.2 and 8.6; Michaelis constant at 37.5°, 0.032 and 0.016; at 24°, 0.034 and 0.018; at 2°, 0.034 and 0.016. The effect of temperature is that predicted by the Arrhenius equation and the heats of activation are 5335 and 11,627 calories, respectively. If the enzyme-substrate compound is identified with the "activated" molecule, the change in free energy will be 1838 and 2264 calories, respectively, and the change in entropy -6.68 and -8.23 calories. The heat of formation of the enzyme-substrate compound is zero in both cases.

The two enzymes differ also in the effect of inhibitors on them. Both are inactivated by acetone but not by alcohol. Cyanide and arsenite affect the liver enzyme more strongly than the jack bean enzyme. Competitive inhibition occurs with metrazol and dimethylhydantoin. The affinity of the former for both enzymes is the same, but the latter has a greater affinity for the liver enzyme than for the other.

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# AN IMPROVED METHOD FOR THE EXTRACTION AND PURIFICATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE\*

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Many methods for preparing diphosphopyridine nucleotide (DPN) suitable for enzymatic studies have been published (1-9). Most of these methods are complicated by a number of different purification steps which are tedious and time-consuming. Sumner and collaborators (7) have proposed a different method of extraction and purification which eliminates the use of heavy metal separations. The latter method was found in this laboratory to yield a crude product of about 1 per cent purity and products of about 30 and 60 per cent purity, respectively, after one and two adsorptions on norit. Although the final product is of a purity similar to that of products obtained by other procedures commonly used (4, 6, 8), the yield is not as high.

The method here described is based on principles found in the original method of Sumner and collaborators, but is modified and improved to give much higher yields of an 85 per cent pure DPN preparation. The five simple and rapid steps which are involved consist of extracting yeast with an acidified mixture of acetone and alcohol, precipitating DPN from this mixture with more alcohol, dissolving the crude DPN in water, and reprecipitating with alcohol, followed by two adsorptions on norit. The final product is a white amorphous material free from gum and completely soluble in water. Enzymatic tests with the TPN<sup>1</sup>-dependent glucose-6-monophosphate system have shown that only about 1 per cent TPN could be present in the final coenzyme preparation.

It is believed that our modified procedure is less difficult to carry out and gives as high as or higher yield and purity of DPN than methods thus far described.

## EXPERIMENTAL

*Extraction and Precipitation of DPN*—Prepare acidified alcohol by cautiously stirring 550 ml. of concentrated H<sub>2</sub>SO<sub>4</sub> into 3500 ml. of cold 95 per cent ethyl alcohol, and cool. Crumble 10 pounds of fresh pressed

\* This work has been made possible by a grant from the Donner Foundation, Inc., whose assistance is gratefully acknowledged.

<sup>1</sup> Triphosphopyridine nucleotide.

bakers' yeast<sup>2</sup> into 3500 ml. of acetone, stir for about 5 minutes, and then add slowly and with stirring the entire volume of acidified alcohol previously prepared. After stirring the suspension (at room temperature) for 15 minutes, it is filtered through large rapid fluted papers or centrifuged.

The clear brown solution (about 6.8 liters by the filtration procedure) is mixed with an equal volume of 95 per cent ethyl alcohol, and the mixture filtered through large fluted papers (Schleicher and Schüll, No. 595). The precipitate, which consists chiefly of  $\text{KHSO}_4$ , is discarded. The filtrate is mixed with another volume of alcohol and is allowed to stand overnight in a cold room at  $-15^\circ$  if possible, or in a refrigerator at approximately  $0-4^\circ$  for 24 to 48 hours. The precipitate is then collected on large fluted filter papers (Schleicher and Schüll, No. 595).

*Reprecipitation of DPN by Alcohol*—The precipitate on the filter papers is collected quantitatively in a total of 200 ml. of distilled water, and an equal volume of 95 per cent alcohol is then added with stirring. The mixture is filtered and 400 ml. of 95 per cent ethyl alcohol are added to the filtrate with stirring. The mixture is placed in the cold room ( $4^\circ$ ) for 12 to 24 hours to obtain separation of a precipitate which can be readily centrifuged.

The precipitated DPN is centrifuged in the cold, and the clear supernatant fluid is discarded. The DPN is suspended in a total of 150 ml. of absolute alcohol and the hard precipitate broken up thoroughly with a stirring rod. The mixture is then centrifuged, the alcohol decanted off, and the precipitate resuspended in 40 ml. of absolute alcohol. After centrifugation, the precipitate is similarly washed twice with 40 ml. portions of ethyl ether. Finally, the DPN is dried, first in air and then *in vacuo* over  $\text{P}_2\text{O}_5$ . The yield at this point is approximately 4 to 4.5 gm. of DPN of 15 to 20 per cent purity. Differences in purity at this stage of the procedure depend to a considerable extent upon the freshness of the yeast used.

*Purification of Crude DPN. First Adsorption*—Dissolve an amount of crude DPN calculated to contain 400 mg. of pure DPN (for example 2.0 gm. of 20 per cent DPN) in 800 ml. of distilled water and stir for 5 minutes with 8 gm. of acid-washed norit (prepared according to Sumner and collaborators (7)). The DPN-norit adsorption complex is centrifuged and washed twice with 800 ml. portions of distilled water and twice with the same volume of dilute ammonia (0.0028 per cent). In washing with ammonia, a considerable length of time is required for centri-

<sup>2</sup> We have used Fleischmann, Anheuser-Busch, and National Grain pressed bakers' yeast as well as top brewers' yeast and have found National Grain yeast to give the highest yield of DPN. However, the freshness of the yeast is probably more important than the particular brand used.

fuging and a small amount of norit is lost. The norit adsorption complex is then washed twice more with distilled water.

The DPN-norit complex is suspended in 100 ml. of distilled water, 10 ml. of isoamyl alcohol are added, and the DPN is eluted from the norit by shaking the mixture mechanically for about 1 hour at room temperature. The norit is then centrifuged and this elution is repeated. The combined eluates are filtered through a wet filter paper to remove any norit which was not completely removed by centrifugation. A third elution will yield only a small additional amount of DPN. The eluates may be stored for at least 24 hours in a refrigerator at 0-4°.

The solution of eluted DPN is made slightly alkaline to phenol red with 1 or 2 drops of approximately 7 N  $\text{NH}_4\text{OH}$ . The solution is then evaporated at reduced pressure with use of a 500 ml. Claisen flask connected to two traps in series, the first of which is cooled with crushed ice and the second with a mixture of acetone and dry ice. The second trap is connected to a vacuum pump. The Claisen flask is equipped with a fine capillary to prevent bumping, and is heated by a water bath held at a temperature of 35-40°. When the volume of solution remaining in the Claisen flask is between 20 and 30 ml., the solution is transferred to a small pear-shaped Claisen flask of about 100 ml. capacity and is further evaporated to a final volume of 2 to 3 ml. The evaporated solution containing the DPN is transferred by pipette to a 50 ml. centrifuge tube and the flask is washed with 2 to 3 ml. of distilled water, this washing being added to the main part of the solution. A volume of absolute alcohol exactly equal to that of the DPN solution (not over 5 ml.) is added to the small Claisen flask to wash out the last traces of DPN. This alcohol washing is then transferred as completely as possible by pipette to the DPN solution in the centrifuge tube. After mixing, the solution is centrifuged to remove any remaining traces of norit. The clear supernatant is transferred to another 50 ml. centrifuge tube and 25 to 30 ml. of absolute alcohol are added. The suspended DPN is then caused to flocculate out by the addition of 2 drops of 7.5 N  $\text{H}_2\text{SO}_4$ . After centrifuging, the clear supernatant is decanted off, and the precipitated DPN is washed with 25 to 30 ml. of absolute alcohol. This alcohol is removed by centrifugation and decantation, and the DPN is then washed once or twice with ethyl ether. The purified DPN is dried in the air and then in a desiccator. The yield at this point is 300 to 400 mg. of DPN with a purity of 65 to 78 per cent. The purity depends upon that of the crude DPN used as starting material; if the latter was approximately 20 per cent pure, the product at this stage should have a purity of 77 to 78 per cent.

In this first adsorption step, the ratio is 50 mg. of 100 per cent DPN to 100 ml. of water to 1 gm. of norit.

*Second Adsorption*—Dissolve an amount of once adsorbed DPN calcu-

lated to contain 400 mg. of pure DPN in 800 ml. of distilled water and mix for 5 minutes with 8 gm. of acid-washed norit. Centrifuge the DPN-norit adsorption complex and wash once with an equal volume (800 ml.) of water, once with the same volume of dilute ammonia (0.0028 per cent), and then once more with the same volume of water.

The DPN is eluted as previously described, except that 5 ml. instead of 10 ml. of isoamyl alcohol are used, and a total of three elutions employed. After making the combined eluates alkaline to phenol red, the solution is again evaporated under a vacuum. The final concentrate (not over 5 ml.) is mixed with an equal volume of absolute alcohol and centrifuged. The clear supernatant is mixed with 30 to 40 ml. of absolute alcohol and 2 drops of 7.5 N  $\text{H}_2\text{SO}_4$  are added. After centrifuging, the precipitated DPN is washed with absolute alcohol and ether as in the previous adsorp-

TABLE I  
*Yield and Purity of DPN at Different Stages of Procedure*

The figures represent ranges obtained from several individual runs starting with 10 pound batches of yeast.

Procedure	Yield	Per cent purity	Calculated pure DPN	Per cent recovery
	mg.		mg.	
Extraction.....	4000-4500	15-20	800-900	
1st adsorption.....	600- 800	65-78	470-620	65-70
2nd ".....	450- 500	85	380-420	65-70

tion procedure, and the product is dried, first in air and then in a vacuum desiccator over  $\text{P}_2\text{O}_5$ . The final, white amorphous product weighs from 250 to 300 mg. and is of approximately 85 per cent purity.

Table I shows the yield and purity of the DPN obtained from 10 pounds of fresh, pressed, bakers' yeast at the three stages of purification already discussed.

**Assay**—The hydrosulfite reduction method of Warburg and Christian (2) as modified by LePage (8) was used to assay the DPN. The absorption of the reduced DPN was measured at 340  $\text{m}\mu$ , from which was deducted the small absorption of an equivalent concentration of oxidized DPN. The Beckman spectrophotometer was employed. The method was found to be satisfactory and reproducible, and compared favorably with the recent modification of Gutcho and Stewart (10).

Since the isolation procedure described had no chemical steps designed to separate DPN from other nucleotides, it seemed necessary to determine the complete ultraviolet absorption spectra of both the oxidized and re-

duced forms of the purified product. These spectra are recorded in Fig. 1, and uniformly resemble those curves for pure DPN which have previously been published (2, 11).

The biological activity of the purified DPN was also tested by measuring the rate and extent of reduction of the oxidized DPN by lactate and lactic dehydrogenase. A comparison was made with a sample of DPN purchased from the Schwarz Laboratories, Inc., which is prepared by a different type of procedure. The enzyme employed was crystalline lactic dehydrogenase, prepared according to Straub's method (12). In order to obtain maximum reduction of DPN (about 75 per cent), it was found necessary to add KCN to combine with the product of the reaction,

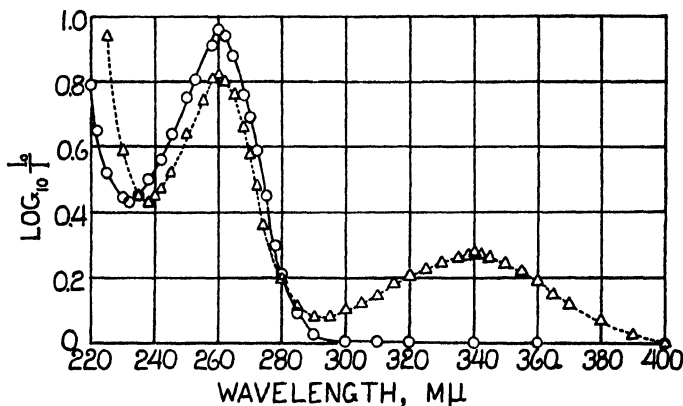


FIG. 1. Absorption spectra of oxidized and reduced DPN. The sample used was a twice adsorbed preparation of 85 per cent purity. ○ oxidized, △ same concentration after hydrosulfite reduction.

pyruvic acid. The reaction system consisted of the following components, added in the order given: 0.50 ml. of 0.10 M  $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  buffer, pH 7.2; 1.10 ml. of distilled water; 1.00 ml. of 0.5 M *dl*-sodium lactate; 0.1 ml. of a diluted solution of crystalline lactic dehydrogenase; 0.2 ml. of 0.2 M neutralized KCN; and 0.10 ml. of an aqueous solution of DPN calculated to contain approximately 0.22 mg. of pure coenzyme. The diluted lactic dehydrogenase was prepared by first dissolving crystals of the enzyme in a small volume of 0.1 saturated  $(\text{NH}_4)_2\text{SO}_4$  to give a strong stock solution (having a concentration of several per cent), and then further diluting with 0.1 saturated  $(\text{NH}_4)_2\text{SO}_4$  to an enzyme concentration convenient for spectrophotometric rate measurements. The control system contained all of the above components except DPN. The light absorption of the reduced form of DPN was measured at 340 mμ in the



Beckman spectrophotometer. The Schwarz Laboratories preparation was treated with  $H_2S$  to remove heavy metal impurity, and was then found to be 55 per cent pure.

In Fig. 2, the rates of enzymatic reduction of the two samples of DPN are compared. It can be seen that the reduction of the Schwarz sample was somewhat slower than that of the sample prepared in this laboratory, although both preparations were finally reduced to the same extent (75 per cent). The different rates of reduction may be due to different amounts of impurities in the samples. Such effects demand consideration in enzymatic studies involving different samples of DPN.

Inasmuch as the calculation of the amount of DPN present is based on the amount of light absorption of the sample at 340  $m\mu$  after reduction

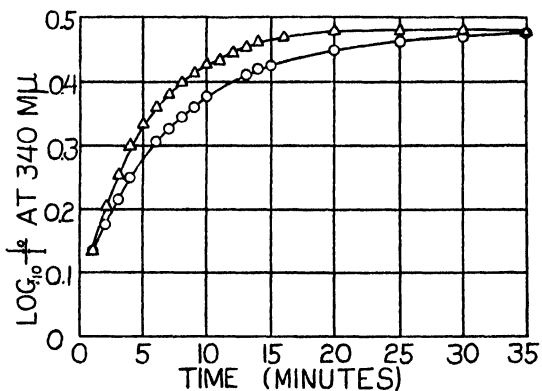


FIG. 2. Reduction of DPN by lactate-lactic dehydrogenase. O preparation from Schwarz Laboratories, 55 per cent purity;  $\Delta$  twice adsorbed DPN, 85 per cent purity.

with hydrosulfite, any triphosphopyridine nucleotide (TPN) in the preparation would be calculated as DPN. The amount of TPN in the DPN preparation was therefore determined from the enzymatic reduction of TPN by Robison ester dehydrogenase and hexose monophosphate.<sup>8</sup> The reaction system was established by adding the following components in the order given: 0.25 ml. of 0.50 M phosphate buffer, pH 7.5; 2.20 ml. of distilled water; 0.20 ml. of enzyme (75 mg. in 3 ml. of 0.25 M phosphate buffer, pH 8.3); 0.25 ml. of 0.10 M sodium glucose-6-phosphate; and 0.10 ml. of a 3 per cent aqueous solution of the purified DPN preparation or of a 1 per cent solution of the purified TPN. The control reaction system contained all of the above components except the coenzyme. The amount of reduced TPN present was determined by measuring the

<sup>8</sup> The authors are indebted to Mr. Kurt Altman for samples of purified TPN and *Zwischenferment*.

amount of light absorption in the Beckman spectrophotometer at 340  $m\mu$  and comparing the reduction obtained with the DPN sample and the known TPN.

By this method, it was found that only about 1 per cent of TPN could have been present in the twice adsorbed (85 per cent pure) product.

#### DISCUSSION

The method described for the preparation of DPN is based on the procedure reported by Sumner and collaborators (7), but three significant changes have been made. The first change consists of an additional fractionation of the crude DPN with alcohol. This step, which can be carried out rapidly, removes excess potassium acid sulfate and other insoluble impurities, thereby allowing for a better degree of purification when the DPN is subsequently adsorbed on norit. It was also found that Sumner and collaborators had used an insufficient amount of acid in the original extraction procedure. Establishment of an optimal acid concentration during extraction enhanced the purity and yield of the crude DPN considerably. The third change is the use of a more effective cytolytic agent,<sup>4</sup> namely acetone, in place of ethyl ether. The use of acetone instead of ether, at optimal acid concentration, together with additional fractional precipitation with alcohol, increased the purity of the crude DPN to values far greater than those of the original method (7), and also increased the yield about 3-fold.

Purification of the crude DPN by adsorption by acid-washed norit can be easily and successfully carried out to obtain a reasonably pure DPN preparation. It was found that, in order to obtain maximum purity and yield in the adsorption steps, it is essential to maintain a proper ratio of DPN to norit. It was also found that the DPN-norit complex should be eluted two or three times with isoamyl alcohol and water in order to recover a reasonably high fraction (70 per cent) of the adsorbed DPN. The elution with isoamyl alcohol and water, originally used by Jandorf (6), was found to be more satisfactory than the elution with pyridine employed by LePage (8).

Attempts to increase the purity of a 65 per cent DPN preparation by the use of lead, silver, or barium precipitation have given no further increase in purity, and instead usually have resulted in a decreased purity. Hence a second adsorption is to be recommended for obtaining DPN of purity greater than 65 per cent. It can be surmised from the work of Hogeboom and Barry (9) that a final purification by means of the technique of counter-current distribution would yield practically pure DPN.

<sup>4</sup> The authors are indebted to Dr. A. Rothstein for his advice in this problem.

## SUMMARY

An improved and comparatively easy method is described for the extraction and purification of diphosphopyridine nucleotide (DPN) from 10 pounds of bakers' yeast with yields of 500 to 800 mg. of approximately 85 per cent pure DPN.

The method is based upon Sumner's procedure (7) of organic solvent extraction and precipitation followed by adsorption on charcoal, without any heavy metal fractionation. This DPN preparation is highly active in biological systems, and contains only a very slight amount of triphosphopyridine nucleotide (TPN).

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# THE EFFECT OF FOLIC ACID UPON THE URINARY EXCRETION OF THE GROWTH FACTOR REQUIRED BY *LEUCONOSTOC CITROVORUM*\*

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In a previous study it was observed that *Leuconostoc citrovorum* 8081 failed to grow on a synthetic medium adequate for the growth of *Leuconostoc mesenteroides* P-60 and other common assay organisms (1). However, the addition to the medium of certain extracts from liver permitted excellent growth of *L. citrovorum*. Similarly, folic acid stimulated growth of the organism but the amounts necessary were very high (1).<sup>1</sup> The present paper is concerned with the effects of folic acid upon the urinary excretion of the *L. citrovorum* factor by the rat.

## EXPERIMENTAL

### Methods

*Care of Animals*—Rats of Alabama Experiment Station strain were placed individually in wire-bottomed cages and fed a basal diet of the composition shown in Table I.

Certain groups of rats received dietary supplements of folic acid, thymine, vitamin B<sub>12</sub> concentrate, sulfaguanidine, or ascorbic acid (see Tables II to IV). Other groups of rats received folic acid by subcutaneous or intraperitoneal injection. In the first series (Tables II and III) the rats averaged 70 gm. and in the second series (Table IV) 200 gm., initial weight. The weight of the animals used had no apparent influence upon the results. Urine collections were made after the rats had been maintained on the respective diets and supplements for a period of at least 1 week. Samples of urine were collected from individual rats over a period of 48 hours. The urine samples were adjusted to pH 6.8, made to a known volume with water, and filtered. The diluted samples were then stored under toluene in a refrigerator until used for the determination of their growth-stimulating activity for *L. citrovorum*.

\* Published with the approval of the Director of the Alabama Agricultural Experiment Station. The folic acid used in this study was donated by the Lederle Laboratories Division, American Cyanamid Company, and all other vitamins, including the vitamin B<sub>12</sub> concentrate, were donated by Merck and Company, Inc.

<sup>1</sup> Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, in press.

*Microbiological Determinations*—The techniques used to determine the activity of rat urine in promoting the growth of *L. citrovorum* were similar to those used by Sauberlich and Baumann (1) in the assay of other crude materials. The only difference was that 5 gm. of acid-hydrolyzed casein supplemented with cystine, cysteine, tryptophan, alanine, and glycine (100 mg. each per 500 ml. of double strength basal medium) were substituted for the amino acids in the medium used by the above investigators. No significant difference was noted in the growth of the organism when this substitution was made.

TABLE I  
*Composition of Basal Diet*

	<i>per cent</i>
Extracted casein (2) . . . . .	20
Corn oil. . . . .	4
Salts Mixture 5 (2) . . . . .	4
Cod liver oil. . . . .	1
Choline chloride. . . . .	0.2
Sucrose to . . . . .	100
	<i>mg. per kg.</i>
Pyridoxine hydrochloride . . . . .	6
Thiamine chloride . . . . .	6
Riboflavin. . . . .	6
2-Methyl-1,4-naphthoquinone . . . . .	2
Nicotinic acid. . . . .	20
Calcium pantothenate . . . . .	30
$\alpha$ -Tocopherol . . . . .	25
$\alpha$ -Tocopherol acetate . . . . .	25
Biotin. . . . .	0.5
Inositol. . . . .	500

In the present study, turbidimetric measurements were used throughout. The results are expressed in terms of *citrovorum* units. 1 *citrovorum* unit was found to be equivalent to 0.025  $\mu$ l. of the liver concentrate, reticulogen (20 U. S. P. units per ml.), and gave a galvanometer reading of about 65 (Evelyn colorimeter, 660  $m\mu$  filter) after an incubation period of 16 to 20 hours (1).

The folic acid content of the urine samples was determined with *Streptococcus faecalis* as the assay organism. The test organism was grown on Medium IV (3) with folic acid omitted, and growth was measured turbidimetrically with the Evelyn colorimeter after an incubation period of 20 to 22 hours.

### Results

Young rats fed the basal diet without supplementary folic acid were found to have an average daily urinary excretion of only 140 *citrovorum* units (Table II). In marked contrast, rats fed the diet supplemented with relatively high levels of folic acid had a high urinary excretion of the factor stimulating the growth of *L. citrovorum*. When the diet contained 10  $\gamma$  of folic acid per gm., the average daily excretion was about 20,000 *citrovorum* units; when the diet contained 5 and 1  $\gamma$  of folic acid per gm., the average daily excretion was about 10,000 and 2000 units, respectively (Table II). In general, the activity of the urine was proportional to the dietary intake of folic acid.

In another series, rats were fed the basal diet containing sulfaguanidine along with supplements of folic acid in an attempt to determine the pos-

TABLE II

*Urinary Excretion of citrovorum Factor by Rat As Influenced by Dietary Folic Acid*

Determinations were made on urine samples collected after the rats had been on the diets for a period of 2 weeks; the results are averages per rat per day.

Folic acid supplement	No. of rats	Folic acid ingested	Folic acid excreted	Excretion, <i>citrovorum</i> units		
				1st wk.	2nd wk.	3rd wk.
$\gamma$ per gm. diet		$\gamma$	$\gamma$			
0	3	0	0.028	74	145	138
0.25	1	2.45	0.126	333	443	
0.5	2	5.85	0.137	205	423	
1.0	1	10.7	0.875	750	1,900	
5.0	2	58.5	1.57	10,300	9,500	
10.0	3	146	6.21	20,300	21,800	23,500

sible effect of intestinal microorganisms upon the production of the factor active for *L. citrovorum*. The results indicated a depression in the activity of the urine when sulfaguanidine was added to the diet (Table III), but the urine still possessed appreciable activity. (Sulfaguanidine did not inhibit the growth of *L. citrovorum* when added in amounts as high as 5 mg. per 10 ml. assay tube; this concentration of sulfaguanidine was much higher than would be encountered in any of the urine samples analyzed.) Furthermore, the administration of folic acid by subcutaneous or intraperitoneal injections resulted in the excretion of relatively active urine (Table III). This would indicate that the intestinal microorganisms were not essential for the excretion of the highly active urines. When the folic acid was injected into the rats in a single dose, the excretion of the *citrovorum* factor in the urine was only one-fourth to one-fifth that

TABLE III

*Excretion of citrovorum Factor by Rat As Influenced by Route of Administration of Folic Acid and by Sulfaguanidine Ingestion*

All rats received the diets or supplements for a period of 2 weeks before urine collections were made; the values are averages of three rats.

Supplements	Route of administration of folic acid	Daily intake of folic acid per rat	Daily excretion of folic acid per rat	Excretion per rat per day
		$\gamma$	$\gamma$	<i>citro- vorum</i> units
None . . . . .		0	0.061	295
1% sulfaguanidine. . . . .		0	0.110	120
10 $\gamma$ folic acid per gm. diet	Diet	150	18.46	34,900
1% sulfaguanidine + 10 $\gamma$ folic acid per gm. diet. . . . .	"	127	10.40	13,500
0.1% thymine . . . . .		0	0.091	202
Folic acid. . . . .	Intraperitoneally	100	33.5	5,000
" " . . . . .	"	500	111.2	11,400
	(1 injection per day)			
" " . . . . .	Intraperitoneally	500	83.0	35,300
	(5 injections daily, 100 $\gamma$ each)			
" " . . . . .	Subcutaneously	100	30.3	3,500
" " + 1% sulfaguanidine. . . . .	Intraperitoneally	100	33.1	4,350

TABLE IV

*Effect of Various Supplements upon Urinary Excretion of citrovorum Factor by Rat*

All rats received the diets or supplements for a period of 1 week before urine collections were made.

Diet	No. of rats	Excretion per rat per day
		<i>citrovorum</i> units
Basal. . . . .	2	309
" + 10 $\gamma$ folic acid per gm. diet. . . . .	3	29,500
" + vitamin B <sub>12</sub> concentrate*. . . . .	2	143
" + 0.1% thymine. . . . .	2	377
" + 0.1% " + vitamin B <sub>12</sub> concentrate*. . . . .	2	248
" + 5 mg. ascorbic acid injected intraperitoneally daily. . . . .	1	100
" + 5 " " " per gm. diet. . . . .	2	235

\*  $\simeq$  0.3  $\gamma$  of vitamin B<sub>12</sub> per gm. of diet.

obtained when an equivalent amount of folic acid was ingested in the diet (Table III). However, if the folic acid was administered to the rats in five separate injections per day instead of in one, the activity of the urine was increased to 35,000 units. The feeding of other supplements that may be related to folic acid metabolism, such as vitamin B<sub>12</sub>, thymine, or ascorbic acid, caused no increase in the activity of the urine above that of rats receiving only the basal diet (Tables III and IV).

Since it is known that high amounts of folic acid will stimulate the growth of *L. citrovorum* (1),<sup>1</sup> it was necessary to assay the urine samples for their folic acid content to determine the probability of folic acid contributing to the activity of the *citrovorum* factor in the samples. From the results of the folic acid analyses of the urine samples (Tables II and III), it may be noted that the urinary excretion of folic acid was directly related to its intake. About 20 to 33 per cent of the folic acid appeared in the urine when the folic acid was administered by injection and only 2 to 12 per cent when the folic acid was added to the diet. This may account for part of the difference in activity of the urine for *L. citrovorum* when the two methods of administering folic acid were used.

For the most part, the total amounts of folic acid in the urine samples were small. In view of the high dilutions necessary for the determination of the *citrovorum* factor in the urine samples, the amounts of folic acid contributed by the samples would be very small. Supplementary experiments showed that the urine samples could contribute amounts of folic acid up to 10  $\gamma$  per 10 ml. assay tube before causing any significant effect on the turbidimetric assay employed for the determination of the growth factor. Thus, even the highest amounts of folic acid found in the urine from rats (Table III) would be well within the limits of safety, since these urine samples would be diluted to such an extent that they would contain not more than 0.01 to 0.02  $\gamma$  of folic acid per ml.

In one experiment, urine samples were collected from a human subject before and after the ingestion of folic acid. The results were similar to those obtained with the rat. Before the ingestion of supplementary folic acid, the subject excreted about 6500 *citrovorum* units per day, but when a single dose of 30 mg. of folic acid was given orally, the urine contained 425,000 units for the following 24 hour period.

#### DISCUSSION

The fact that urine from the rat or man fed relatively large amounts of folic acid is highly active in stimulating the growth of *L. citrovorum* indicates either that animals are capable of converting folic acid to a more active compound or that the added folic acid stimulates the excretion



of an active compound. The resulting product may be the metabolically active form of folic acid for animals or only an excretory product of folic acid metabolism.

The evidence indicates that the intestinal bacteria are not essential for the production of the active principle. Folic acid injected subcutaneously or intraperitoneally still permitted the excretion of a highly active urine. Furthermore, when folic acid was administered in five injections per day, the activity of the urine was increased considerably over that obtained when the folic acid was given in a single injection per day. This suggests that the rate of absorption determines the efficiency of conversion of folic acid to the active principle. The excretion of folic acid in the urine would also suggest this relationship. Experiments on the human have indicated that about 15 to 50 per cent of the ingested or administered folic acid could be accounted for in the urine (4-9). Similar results were obtained in this investigation with rats. Perhaps part of the apparent disappearance of folic acid may be accounted for in the urine as the factor active for the growth of *L. citrovorum*.

Recent reports indicate close relationships between vitamin B<sub>12</sub>, thymidine, folic acid, and possibly ascorbic acid (10-19). For instance, thymidine has been shown to be capable of replacing the vitamin B<sub>12</sub> requirement of *Lactobacillus lactis* Dorner (12, 14, 15) and *Lactobacillus leichmannii* (15-17). Moreover, Shive *et al.* (15) have reported that thymidine, after a lag phase, slowly replaces both the folic acid and the vitamin B<sub>12</sub> required for the growth of *L. leichmannii*. Similarly, Shive *et al.* (11) have demonstrated that the inhibition of growth of *Leuconostoc mesenteroides* 8293 by methylfolic acid is overcome more readily with thymidine than with folic acid. Thymidine to a limited extent also replaces the factor required by *L. citrovorum*, but vitamin B<sub>12</sub> appeared to be inactive (1).<sup>1</sup> Vitamin B<sub>12</sub> has been postulated to be involved in the synthesis of thymine or its conversion to thymidine (14, 15). The results with rats fed vitamin B<sub>12</sub> concentrates, thymine, or vitamin B<sub>12</sub> concentrates and thymine together do not indicate any significant increases in the activity of the urine, although the excretion of thymidine to any great extent in the urine would have been detected by *L. citrovorum*. Similarly, rats fed ascorbic acid showed no increased activity in the urine.

The possibility remains, however, that folic acid stimulates the production and excretion of thymidine. In this respect it should be noted that the response of *L. citrovorum* to graded amounts of the urine samples resulted in linear relationships that were not obtained when pure thymidine was tested (1).<sup>1</sup> This indicates that the active compound in the urine is not thymidine.

## SUMMARY

1. Rats fed diets without supplementary folic acid excreted urine that was low in the factor required by *Leuconostoc citrovorum* 8081. However, rats fed diets containing 1 to 10  $\gamma$  of folic acid per gm. excreted urine that was very high in this factor, the activity being proportional to the amounts of folic acid ingested. The activity was increased as much as 200-fold on the higher levels of folic acid.

2. The presence of sulfaguanidine in the diet suppressed somewhat the excretion of the active principle; however, folic acid administered to rats subcutaneously or intraperitoneally resulted in a relatively active urine. The feeding of thymine, vitamin B<sub>12</sub>, or ascorbic acid did not increase the activity of the urine.

3. The ingestion of 30 mg. of folic acid by a human subject markedly increased the urinary excretion of the *L. citrovorum* factor.

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# CONVERSION OF OCTANOIC ACID TO RAT LIVER GLYCOGEN, STUDIED WITH C<sup>14</sup>,C<sup>13</sup>-LABELED OCTANOATE\*

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In a study of the conversion of isotopic acetate and butyrate to liver glycogen in the intact rat, it was shown that isotope from the carboxyl group of acetate appears primarily in carbons 3 and 4 of the glucose derived from the liver glycogen (1). The label from the methyl carbon of acetate was found predominantly in positions 1, 2, 5, and 6 of the glucose. Carboxyl- and  $\beta$ -labeled butyrate yielded the same isotope distribution pattern as found with carboxyl-labeled acetate, while  $\alpha$ -labeled butyrate gave rise to an isotopic glycogen similar to that following administration of methyl-labeled acetate. These findings are consistent with  $\beta$  oxidation of butyrate to 2 molecules of acetate, or an acetate-like intermediate.

In the present experiments liver glycogen has been employed in an effort to study the metabolism of a fatty acid of intermediate chain length in the intact animal. Octanoate containing C<sup>13</sup> in the carboxyl group and C<sup>14</sup> in the 7-( $\gamma$ )carbon has been used. This type of labeling affords an opportunity to study the fate of the carboxyl and  $\gamma$ -carbons under strictly comparable conditions. If octanoate is metabolized via  $\beta$  oxidation to acetate-like 2-carbon units, the first and last of these fragments should be carboxyl-labeled, and both types of isotope should appear principally in carbons 3 and 4 of the glucose from the liver glycogen (1). This has been found to be the case. However, the contribution of the carboxyl carbon to positions 3 and 4 of the glucose has been found to exceed that of the  $\gamma$ -carbon by about 30 per cent. The possible significance of this finding will be considered.

## Methods

C<sup>13</sup>- and C<sup>14</sup>-labeled octanoate were prepared separately, and mixed together prior to use. *n*-Octanoic acid-carboxyl C<sup>13</sup> was synthesized by the Grignard reaction. *n*-Octanoic acid-7-C<sup>14</sup> was made by a synthesis similar in principle to that employed by Dauben in the preparation of palmitic acid-6-C<sup>14</sup> (2). C<sup>14</sup>-carboxyl-labeled acetate was reduced to

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ethanol with lithium aluminum hydride, then converted to the bromide, and the diethyl cadmium salt prepared via the Grignard reagent. This was condensed with the ethyl ester chloride of adipic acid, hydrolysis of the condensation product yielding 6-keto-*n*-octanoic acid. The keto acid was reduced by a modified Wolff-Kishner reaction to form *n*-octanoic acid-7-C<sup>14</sup>. The C<sup>14</sup>-labeled acid was purified by repeated steam distillation and recrystallization from cold water. Both products exhibited the same boiling and melting points as did authentic octanoic acid.

Two 24 hour fasted male albino rats were fed 1.1 mm of octanoate and 3.38 mm of glucose, per 100 gm. of body weight, by stomach tube. Respiratory CO<sub>2</sub> was collected throughout the experiment. After 3 hours, liver glycogen was isolated, hydrolyzed to glucose, and degraded. The methods used were those previously reported (1, 3, 4).

#### RESULTS AND DISCUSSION

The general experimental data are summarized in Table I. In Table II are presented the results obtained from the degradation of the liver glycogen.

The appearance of isotope predominantly in carbons 3 and 4 of the glucose is consistent with conversion of octanoate to glycogen via 2-carbon fragments (1). However, it should be pointed out that, on the basis of available knowledge concerning acetoacetate metabolism (5), condensation of the 2-carbon units to acetoacetate prior to conversion to glycogen would not be expected to affect the isotope distribution pattern. The traces of isotope found in the other positions of the glucose are of the same order of magnitude as previously reported in CO<sub>2</sub> fixation experiments (4). A significant difference is apparent in the relative contributions of the carboxyl and  $\zeta$ -carbons of octanoate to the 3 and 4 positions of the glucose, the carboxyl carbon predominating in a ratio of approximately 1.3:1. Assuming that the principal pathway for the transfer of carbon to glycogen from 2-carbon fragments arising from fat catabolism is the tricarboxylic acid cycle (1), it may be inferred from the foregoing result that the terminal 2-carbon unit from octanoate, because of the presence of a methyl group, reacts less rapidly in the initial condensation reaction of the cycle than the 2-carbon fragments arising from the rest of the chain. The results of experiments on the conversion of isotopic octanoate to acetoacetate in liver homogenates have led Gurin and Crandall (6) to postulate a difference in reactivity of the terminal 2-carbon unit of octanoate in acetoacetate formation.

The predominance in the glycogen of the carboxyl over the  $\zeta$ -carbon may be explained in other ways. Since the isotopic composition of liver glycogen may be assumed to reflect hepatic metabolism primarily, the lower

$C^{14}$  content might result from a greater extrahepatic metabolism of the terminal carbons of the fatty acid chain. This could be achieved if more terminal than carboxyl 2-carbon units were converted to acetoacetate or if the last 4 carbons of part of the octanoate were converted directly to acetoacetate, since acetoacetate appears to be metabolized primarily in extrahepatic tissues. Available evidence indicates that the first 4 carbons

TABLE I  
*General Experimental Data*

Experiment No.	Body weight after fasting	Type of octanoate administered	Amount of octanoate administered	Amount of glucose administered	Liver weight	Liver glycogen (as glucose)
	gm.		mm per 100 gm. body weight	mm per 100 gm. body weight	gm.	mm
1	180	$CH_3 \cdot C^{14}H_2 \cdot (CH_2)_4 \cdot C^{13}OONa$	1.1	3.38	6.36	1.39
2	175	"	1.1	3.38	7.44	1.79

TABLE II  
*Distribution of Isotope in Liver Glycogen after Administration of  $C^{14}$ ,  $C^{13}$ -Labeled Octanoate*

Experiment No.	Type of isotope	Isotope concentration* in labeled carbon of administered octanoate	Isotope concentration† in degradation fractions of glucose			Per cent administered isotope recovered in carbon atoms 3 and 4
			Carbon atoms of glucose			
			3, 4	2, 5	1, 6	
1	C <sup>13</sup>	17.2	1.92	0.06	0.06	2.69
	C <sup>14</sup>	39,700	1.49	0.05	0.06	2.09
2	C <sup>13</sup>	17.2	2.09	0.06	0.06	3.89
	C <sup>14</sup>	39,700	1.58	0.04	0.06	2.93

\* Expressed as atom per cent excess for  $C^{13}$ , and counts per minute per mg. of carbon for  $C^{14}$ .

† Expressed as per cent of the isotope concentration in the labeled carbon of the administered octanoate.

of octanoate are not transformed directly to acetoacetate, but proceed via 2-carbon fragments (6). Direct conversion, however, has not been excluded for the terminal 4 carbons.

The data on the respiratory  $CO_2$ , presented in Table III, show that the carboxyl and  $\gamma$ -carbons of octanoate enter the respiratory  $CO_2$  at identical rates, in contrast to the finding in the liver glycogen. It might be expected that the isotope occurring in the glycogen in relatively higher concentration would be relatively less abundant in the respiratory  $CO_2$  simply because less was available for transformation to  $CO_2$ . However, the per cent

recovery of administered isotope in the glycogen is so small compared to that in the respiratory  $\text{CO}_2$  that the expected difference could not be detected reliably. Although the finding of equal amounts of carboxyl and  $\beta$ -carbon in the respiratory  $\text{CO}_2$  raises other questions concerning the interpretation of the glycogen data, it should be borne in mind that, while the glycogen may be considered as reflecting hepatic metabolism, the respiratory  $\text{CO}_2$  arises from all the tissues. The difference noted between the glycogen and respiratory  $\text{CO}_2$  may therefore be indicative of different metabolic patterns in liver and peripheral tissues. On the other hand, it may mean that the pathways for the transfer of carbon from fatty acids to carbohydrate and to  $\text{CO}_2$  are not identical, as previously assumed (1).

The present experiments throw some light on the possible rôle of  $\omega$  oxidation in octanoate metabolism in the liver. If  $\omega$  oxidation, followed by  $\beta$

TABLE III  
*Data on Respiratory  $\text{CO}_2$*

Experiment No.	$\text{CO}_2$ output in mm			Type of isotope	Isotope concentration* in respiratory $\text{CO}_2$			Per cent administered isotope recovered in respiratory $\text{CO}_2$
	1st hr.	2nd hr.	3rd hr.		1st hr.	2nd hr.	3rd hr.	
1	10.65	8.97	8.05	$\text{C}^{13}$	2.73	3.28	3.20	42.6
				$\text{C}^{14}$	2.70	3.23	3.26	42.5
2	10.41	9.59	8.97	$\text{C}^{13}$	2.96	3.78	3.17	49.5
				$\text{C}^{14}$	2.97	3.79	3.16	49.6

\* Expressed as per cent of the isotope concentration in the labeled carbon of the administered octanoate.

oxidation, occurred with the formation of  $\text{C}^{14}\text{H}_3\cdot\text{COOH}$  or  $\text{COOH}\cdot\text{C}^{14}\text{H}_2\cdot\text{CH}_2\cdot\text{COOH}$ , the  $\text{C}^{14}$  introduced into liver glycogen by this mechanism should reside chiefly in carbons 1, 2, 5, and 6. The results indicate that such a mechanism, if present, is quantitatively trivial.

Finally, a word should be said about the isotope found in positions 1, 2, 5, and 6 of the glucose. In the present experiments these carbons are found to have 2 to 4 per cent of the isotope content of positions 3 and 4. This relationship has been noted previously in  $\text{CO}_2$  fixation experiments (4) and has been confirmed by Gibbs (7). There are no well established pathways to account for the introduction of isotope into these positions, either from  $\text{CO}_2$  or the carboxyl group of a 2-carbon unit. An attempt to explain this finding has been made in a prior communication (4). The possibility exists that the label appearing in positions 1, 2, 5, and 6 in the present experiments and in the  $\text{CO}_2$  fixation experiments may be an artifact

of the degradation procedure perhaps introduced during the bacterial fermentation of the glucose.

#### SUMMARY

C<sup>14</sup>, C<sup>13</sup>-labeled octanoate ( $\text{CH}_3 \cdot \text{C}^{14}\text{H}_2 \cdot (\text{CH}_2)_5 \cdot \text{C}^{13}\text{OONa}$ ), together with glucose, was administered to fasted rats. The distribution of isotope in the resulting liver glycogen was found to be consistent with octanoate metabolism via 2-carbon fragments arising by  $\beta$  oxidation. The two isotopes entered the respiratory  $\text{CO}_2$  at identical rates, but relatively more C<sup>13</sup> than C<sup>14</sup> appeared in the liver glycogen. The possible significance of this finding has been discussed.

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# ON NAPHTHOQUINONES AS INHIBITORS OF SPORE GERMINATION OF FUNGI\*

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Reports on the antifungal activity of naphthoquinones have been concerned chiefly with their action on animal pathogens. However, 2,3-dichloro-1,4-naphthoquinone has gained commercial importance as a plant fungicide (1), and this laboratory has reported the activity of 2-methoxy-1,4-naphthoquinone (2) and 2-methylmercapto-1,4-naphthoquinone (3) against certain plant pathogens. These findings directed our interest to a study of the point of action of naphthoquinones as inhibitors of spore germination.

Data are presented to show that certain naphthoquinones, which are not substituted by hydroxyl in positions 2 and 3, are potent inhibitors of the carboxylase system.

A general parallelism was found to exist between the effective dose necessary to produce 50 per cent inhibition of spore germination ( $ED_{50}$ ) (4) with *Monilinia fructicola* and the quantity required to produce 50 per cent inhibition of carbon dioxide output by the carboxylase system. This suggests that a possible locus of antifungal action of certain naphthoquinones is this enzyme.

If this were so, it was reasoned that the enzyme system might provide a means of determining the antifungal potency of naphthoquinones and other compounds.

## EXPERIMENTAL

The naphthoquinones employed were synthesized in this laboratory by procedures reported in the literature. 2-Cyclohexyl-3-hydroxy-1,4-naphthoquinone was supplied by Dr. L. F. Fieser. The required concentrations were dissolved in  $H_2O$  and checked with a Beckman spectrophotometer. The quantities necessary to produce 50 per cent inhibition are reported as the concentration in the final enzyme-substrate-inhibitor mixture.

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A study was first made of the effect of naphthoquinones on the respiration and fermentation of a living organism. *Saccharomyces cerevisiae* was chosen for this preliminary work because of availability and ease of manipulation. A suspension of washed cells was prepared from bakers' yeast (Fleischmann). The concentration of cells was so adjusted that the oxygen consumption of the control was approximately 60  $\mu$ l. per hour. Glucose (10 per cent solution) was employed as the substrate and 0.5 M acetate buffer at pH 4.8 as the suspending medium. The temperature of the bath was kept at 28°.

The direct method of Warburg was used for the measurements of carbon dioxide and oxygen. Readings were taken for 30 minutes in the absence of inhibitor, after which a solution of the naphthoquinone was added from the side arm and readings continued for 2 hours.

The carboxylase was prepared from bakers' yeast (5) according to the method of Green, Herbert, and Subrahmanyam (6). The product of the third fractionation with ammonium sulfate, designated by the authors (6) as Fraction IIIb, was used as the purified enzyme. This was suspended in sufficient half saturated ammonium sulfate so that 0.2 cc. of the enzyme in the final fluid volume of 3.3 cc. in the manometer cup would produce about 250  $\mu$ l. of carbon dioxide per hour.

The side arm of the Warburg vessels contained 0.5 cc. of 0.25 M sodium pyruvate (as substrate) adjusted to pH 6. The main vessel contained 0.3 cc. of 0.2 M citrate buffer at pH 6, 0.2 cc. of the purified carboxylase preparation; a solution of the substances to be tested, and water to give a final volume of 3.3 cc. The gas phase was air and the temperature was maintained at 30°.

The evolution of gas for the second half hour after tipping in the substrate was taken as a measure of the activity.

### Results

The effect of 2-methoxy-1,4-naphthoquinone as the inhibitor of metabolism of yeast is shown in Fig. 1. It is evident that there is a slight stimulation of respiration for the first 90 minutes. (This effect is somewhat more pronounced at lower concentrations of the inhibitor.) Gaffron (7) has noted a similar effect upon the respiration of algae by appropriate concentrations of some quinones, and attributes it to a catalysis of the oxidation of material already present in the cells. Accompanying this slight increase in oxygen consumption is a pronounced decrease in evolution of carbon dioxide. Since certain naphthoquinones have been found to be potent inhibitors of carboxylase (8), it seemed that the fermentation of yeast might be blocked at this point. Similarly, the naphthoqui-

nones might be effective at this point in inhibiting the germination of fungus spores.

Table I records the relative potencies of several naphthoquinones re-

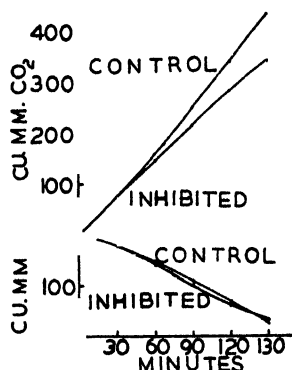


FIG. 1. The effect of  $106 \times 10^{-6}$  M 2-methoxy-1,4-naphthoquinone on the metabolism of yeast. Inhibitor added after 30 minutes.

TABLE I

Molar Concentrations of Various Compounds Required to Produce 50 Per Cens Inhibition

Compound	$ED_{50}$ against carboxylase	$ED_{50}$ against <i>Monilinia fructicola</i>
2,3-Dichloro-1,4-naphthoquinone.....	$2.86 \times 10^{-7}$	$3.1 \times 10^{-7}$
2-Amino-1,4-naphthoquinonimine HCl.....	$2.88 \times 10^{-6}$	$5.34 \times 10^{-6}$
2-Mercapto-1,4-naphthoquinone.....	$4.8 \times 10^{-6}$	$5.0 \times 10^{-6}$
1,4-Naphthoquinone.....	$4.24 \times 10^{-6}$	$62.0 \times 10^{-6}$
2,3-Dimethoxy-1,4-naphthoquinone ..	$46.3 \times 10^{-6}$	$22.8 \times 10^{-6}$
2-Methoxy-1,4-naphthoquinone.....	$106 \times 10^{-6}$	$16.4 \times 10^{-6}$
2-Amino-1,4-naphthoquinone.....	$205 \times 10^{-6}$	$52.5 \times 10^{-6}$
2-Hydroxy-1,4-naphthoquinone.....	$701 \times 10^{-6}$	$>600 \times 10^{-6}$
J-49*.....	$19.6 \times 10^{-6}$	$119 \times 10^{-6}\dagger$
2K-5*.....	$36.7 \times 10^{-6}$	$140 \times 10^{-6}\dagger$
36-L*.....	$264 \times 10^{-6}$	$2000 \times 10^{-6}\dagger$

\* Substituted pyrazoles supplied by the Naugatuck Chemical Company.

†  $LD_{50}$  on tomato foliage infected with *Alternaria solani* (McNew, G. L., and Sundholm, N. R., unpublished data).

ported as the molar concentration required to produce 50 per cent inhibition of carbon dioxide output. For comparison the  $ED_{50}$  values (4) against *Monilinia (Sclerotinia) fructicola* are also shown for some of the compounds. Although obvious discrepancies exist, there is better agree-

ment among those quinones which inhibit at very low concentrations and are therefore possibly more specific.

The greater activity of 1,4-naphthoquinone *in vitro* is not surprising in view of its greater reactivity. The possibility of combination with proteins other than the enzyme protein is thus enhanced and might serve to lower its effectiveness *in vivo* (9). A similar effect was noted by Bueding, Peters, and Waite (10) in the inhibition of glycolysis of *Schistosoma mansoni* by 1,4-naphthoquinone and is comparable to the serum inactivation of many antibiotics.

The loss of activity with the introduction of a hydroxyl group is paralleled in the test of spore germination. Oxford (11) found that the intro-

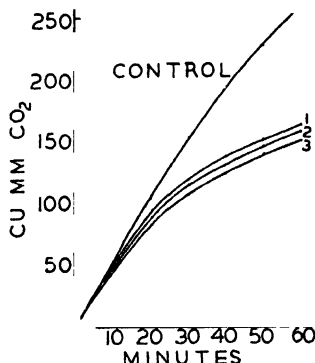


FIG. 2. The effect of prior time of contact of inhibitor ( $3.6 \times 10^{-4}$  M 2-amino-1,4-naphthoquinonimine hydrochloride) with components of the enzyme system. Curve 1, substrate and inhibitor for 80 minutes; Curve 2, enzyme and inhibitor for 20 minutes; Curve 3, enzyme and inhibitor for 80 minutes.

duction of hydroxyl into benzoquinone derivatives similarly resulted in a decrease in antibacterial activity of those compounds. On the other hand, Ball *et al.* (12) reported that certain 2-hydroxy-3-alkylnaphthoquinones were very potent general respiratory inhibitors. Several of the naphthoquinones inhibit the oxygen uptake of the succinate oxidase system and the respiration of the malarial parasite, *Plasmodium knowlesi*. 2-Hydroxy-3-cyclohexyl-1,4-naphthoquinone, which was found by Fieser and Heymann (9) to inhibit the respiration of red blood cells parasitized with *Plasmodium lophurae*, was completely inactive against the carboxylase system. Methylation of the hydroxyl group resulted in a compound which was active against the enzyme but was too insoluble to produce 50 per cent inhibition of carbon dioxide evolution.

The possibility that inhibition of carboxylase might provide a quick and reliable guide to antifungal activity was investigated. Assuming the

activity of any one class of compounds against the enzyme, the results indicate that the test *in vitro* affords a means of revealing the more potent members thereof. Other naphthoquinones, which are inhibitors of spore germination but for which no  $ED_{50}$  was obtained, are also inhibitors of carboxylase. In the pyrazole series (Table I), the values obtained for Compounds 2K-5 and J-49 against the enzyme were decidedly lower than the value for Compound 36-L. This is paralleled in the field tests and in the  $ED_{50}$ .<sup>1</sup> Certain dyes, such as methylene blue and malachite green which are antifungal (13), were found to inhibit carboxylase. Tetramethylthiuram disulfide and disodium ethylenebisdithiocarbamate gave inconsistent results. This may be due to insolubility of these compounds, a limiting factor in this type of assay.

Of theoretical interest was the observation that no great inhibition of carboxylase occurred unless the enzyme was in contact with the substrate. No appreciable difference was seen between the experiment in which the substrate was added immediately after equilibration and that in which the enzyme and inhibitor were in contact 1 hour longer before tipping in the substrate. To rule out a reaction between inhibitor and substrate, these compounds were allowed to remain in contact for 80 minutes, after which the enzyme was added from the side arm. These results are shown in Fig. 2.

The inhibition of carboxylase by naphthoquinones may therefore be classified as non-competitive with the inhibitor, combining only with the enzyme-substrate intermediate. This was confirmed by application of the equations outlined by Ebersole *et al.* (14) for the kinetic analysis of the various types of inhibition.

#### DISCUSSION

The work of Wendel (15) and of Heymann and Fieser (16) has shown that proteins depress the activity of naphthoquinones and that the ratio of susceptibility to different proteins is by no means constant. Other factors, such as diffusibility into the living cell and the differences in time that the inhibitor is in contact with the living and the enzyme systems, would tend to produce varying results. In view of this, the close agreement between values *in vitro* and *in vivo* for the most potent compounds and the rough parallelism of the other results would seem to be significant. The finding that the introduction of a hydroxyl into the naphthoquinone nucleus markedly decreased its anticarboxylase activity as well as its activity against spore germination could be taken as further evidence for this hypothesis. This does not imply, however, that the carboxylase system is the sole point of inhibition of the antifungal naphthoquinones,

<sup>1</sup> McNew, G. L., and Sundholm, N. R., unpublished data.

since 2-hydroxy-3-cyclohexyl-1,4-naphthoquinone inhibits the germination of spores, possibly through inhibition of succinoxidase.

It has not been possible to ascertain the manner in which naphthoquinones block the carboxylase system. The fact that the enzyme must be in contact with the substrate to afford inhibition might provide an approach to the problem. Carboxylase is a sulfhydryl-containing enzyme (17). It is known that sulfhydryl groups differ in availability, possibly depending upon whether they are superficial or deep in the protein (18, 19). If, therefore, the inhibitory action of naphthoquinones involves the sulfhydryl groups, the possibility exists that it is the so called sluggish sulfhydryl groups which are affected and become available only when the enzyme is functioning.

Studies are being undertaken in an attempt to clarify the observation that the three components must be present.

#### SUMMARY

1. Certain hydroxyl-free naphthoquinones and other compounds have been shown to be active against carboxylase.

2. Parallelism exists between quantities necessary to produce 50 per cent inhibition against spore germination of *Monilinia fructicola* and of the activity of carboxylase. This relationship suggests that carboxylase is one of the points of inhibition of antifungal naphthoquinones.

3. This activity might provide a quick and reliable guide for testing certain antifungal compounds.

4. The observation that the enzyme must be functioning in order to be inhibited is made. Possible implications of the phenomena are discussed.

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# THE EFFECT OF CARBOHYDRATE ON THE OXIDATION OF FATTY ACIDS BY LIVER SLICES\*

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Although the mechanism of ketone body formation from fatty acids is now well established (1-3), the factors which regulate their production by animal cells remain undisclosed. One widely quoted explanation for the antiketogenic action of carbohydrate is that carbohydrates and fatty acids are in competition for the oxygen available to the normal liver cell, and that when both substrates are present carbohydrate is utilized in preference to fatty acids (4-6). According to this concept ketogenesis is a reflection of an increased fatty acid catabolism when carbohydrate is lacking or cannot be utilized.

Supporting evidence for this hypothesis stems mainly from studies of Edson *in vitro* (7) who found a generally lower endogenous ketogenesis by liver slices from nourished than from fasted animals, and also found many oxidizable substrates, such as glucose, glycerol, and lactate (8) inhibited ketogenesis to varying extents. Further support for this view was provided by Cohen (9) who considered the antiketogenic effect of carbohydrate to be a result of lowered fatty acid catabolism due to competition for the oxidative enzyme surfaces between structurally similar fatty acid and carbohydrate intermediates.

The availability of isotopically labeled fatty acids has made possible an experimental appraisal of this concept of ketogenesis. It is difficult ordinarily with liver slices to determine the rate of oxidation of a metabolite by the differences in oxygen uptake from endogenous levels because of the already high endogenous respiration and the uncertainty concerning the effect of the added metabolite on the endogenous metabolism. With isotopic substrates, however, the rate of oxidation can be determined, regardless of the magnitude of endogenous respiration, simply by measurement of the amount and isotopic content of the respiratory  $\text{CO}_2$ .

This procedure was employed in the present study to ascertain the effect

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† Taken in part from the thesis of Bernice Friedman in candidacy for the degree of Master of Arts in the Graduate School of Temple University.

of carbohydrate on the rates of oxidation of fatty acids by surviving liver slices. The oxidation rates of a series of acids ranging from acetic to octanoic were compared in (1) the glycogen-"free" liver of the fasted rat, (2) the glycogen-rich liver of the nourished rat, and (3) the liver of the fasted rat in the presence of the easily oxidizable carbohydrate intermediate, pyruvic acid. The results of this study provided no support for the idea that carbohydrate is oxidized by liver preferentially to fatty acids.

### Methods

*Isotopic Substrates*—The fatty acids used in this study were labeled in the carboxyl position with carbon 13, having been prepared by the action of isotopic  $\text{CO}_2$  on the appropriate Grignard reagent. The carboxyl  $\text{C}^{13}$  excesses were as follows: octanoic acid 9.60, hexanoic acid 7.67, butyric acid 7.41, and acetic acid 5.53, representing over-all excesses, respectively, of 1.20, 1.28, 1.85, and 2.77 atom per cent.

*Experimental Procedure*—The liver slices were incubated in 250 ml. Warburg type flasks each carrying a side bulb and a 24/40 standard taper joint. These were attached to ordinary Warburg manometers, the ground joints of which were replaced by 24/40 standard taper joints through which extended an inner tube. To this inner tube there was attached a filter paper roll soaked with  $\text{CO}_2$ -free KOH for absorption of the respiratory  $\text{CO}_2$ . This arrangement made it possible to measure the oxygen consumption and to collect the respiratory  $\text{CO}_2$  in a single experiment. Though oxygen consumption measurements were not very precise, they served to indicate whether respiration was proceeding normally.  $Q_{O_2}$  values ranged from 6 to 14; these are of the same order as in comparable microscale experiments (7-10).

The animals used in this study were stock colony male rats fed Purina dog chow, weighing approximately 200 gm. The fasted rats received no food for 24 hours prior to an experiment and had a liver glycogen content of 0.06 to 0.2 per cent, as compared with values of 2 to 4 per cent for the nourished rats.

They were killed by decapitation, and, after removal of a sample for glycogen assay (11), the liver was sliced with the Stadie-Riggs slicer (12). Approximately 5 gm. were weighed and then distributed equally between two flasks, each containing 30 ml. of phosphate-buffered saline solution (13) and the substrates in 0.01 M concentration. The flasks were filled with oxygen and shaken for 2 hours at 37°, after which sufficient acid was tipped in from the side bulb to release bound  $\text{CO}_2$ . After an additional 5 to 10 minutes shaking, the flasks were cooled; the filter paper rolls were quickly transferred to a flask containing barium chloride solution, and the precipitated barium carbonate filtered off under nitrogen. Two known

sources of error are involved in this procedure; there is a small blank correction for reagents, amounting to about 10  $\mu\text{M}$  of  $\text{CO}_2$ , and there is also a slow decomposition of acetoacetate, amounting to about 3 to 5 per cent per hour (14)<sup>1</sup> which, in these experiments would account for another 5 to 10  $\mu\text{M}$  of  $\text{CO}_2$ . Since these errors are rather small, and are probably balanced to some extent by unknown losses, we have not applied any corrections.

The solutions were decanted from the slices, the latter were washed several times, and the combined solutions and washings acidified with sulfuric acid and distilled with steam. The distillate, containing unchanged acid, together with acetone derived by thermal decarboxylation of the acetoacetate, was caught in an iced receiver, neutralized, and redistilled, thereby separating the acetone from the residual fatty acid. The acetone was isolated as the Denigès complex (15) and the acids determined and recovered by the procedure of Friedemann (16).

*Calculations*—For  $\text{C}^{13}$  determinations, the respiratory  $\text{CO}_2$  was liberated by action of acid, and the acetone-mercury complexes oxidized to  $\text{CO}_2$  by wet combustion (17). The isotope excesses of the respiratory  $\text{CO}_2$  are computed as relative values, based on an assumed 100 per cent over-all excess in the fatty acid; *i.e.*, relative  $\text{C}^{13}$  excess = actual  $\text{C}^{13}$  excess  $\times$  100/ $\text{C}^{13}$  excess of fatty acid.

In the first experiment of Table I, for example, the respiratory  $\text{CO}_2$  had an excess of 0.58 atom per cent. Since the octanoate had an over-all excess of 1.20 per cent, the relative  $\text{C}^{13}$  excess of the respiratory  $\text{CO}_2$  is  $0.58 \times 100/1.20 = 48$  per cent. This value is recorded in Column 3. On the reasonable assumption that the labeled carboxyl carbon of the fatty acid is converted to  $\text{CO}_2$  to the same extent as the other 7 carbons, that is, that the labeled position is representative of all the fatty acid carbon, the relative isotope excess gives the actual proportion of fatty acid carbon in the respiratory  $\text{CO}_2$ . On this basis 48 per cent of all the respiratory  $\text{CO}_2$  can be considered to represent octanoate carbon. Since a total of 309  $\mu\text{M}$  of respiratory  $\text{CO}_2$  was obtained, (Column 2) we can calculate that  $309 \times 0.48 = 149$  microatoms of octanoate carbon were completely oxidized. This is the value given in Column 4. Calculated in this way, this value expresses the capacity of a tissue to oxidize a fatty acid regardless of its chain length.

*Over-All  $\text{C}^{13}$  Excess of Acetoacetate*—In those experiments in which octanoate, hexanoate, and acetate were used as substrates, the over-all  $\text{C}^{13}$  excess of the acetoacetate was computed from the  $\text{C}^{13}$  excess of the Denigès complex on the assumption that all of the excess  $\text{C}^{13}$  of the acetone was in the carbonyl carbon and that this position, representing the acetoacetate  $\beta$ -carbon, had the same  $\text{C}^{13}$  excess as the acetoacetate carboxyl carbon.

<sup>1</sup>Also our own unpublished observations.

For example, in the first experiment of Table II, the acetone had a  $C^{13}$  excess of 0.60 per cent, representing  $0.60 \times 3 = 1.80$  per cent in the carbonyl carbon. This represents an over-all  $C^{13}$  excess in the 4 acetoacetate carbons of  $1.80 \times 2/4 = 0.90$  per cent. Since the octanoate had an over-all  $C^{13}$  excess of 1.20 per cent, the relative  $C^{13}$  excess is  $0.90 \times 100/1.20 = 75$  per cent; that is the value given in Column 3.

These calculations are based on previous studies (1) which disclosed that the acetoacetate formed from carboxyl-tagged octanoic acid had an essen-

TABLE I

*Yields and  $C^{13}$  Excesses of Respiratory  $CO_2$  from Oxidation of Carboxyl-Labeled Fatty Acid by Rat Liver Slices*

The substrates were used in 0.01 M concentration, and incubation was carried out for 2 hours at  $37^\circ$  in oxygen. Calculated for 1 gm. dry weight of tissue; quantities in micromoles;  $C^{13}$  values in atom per cent excess, based on 100 per cent excess in the fatty acid.

Acid (1)	Fasted			Fed			Fasted + pyruvate, 0.01 M		
	Total (2)	Relative $C^{13}$ excess (3)	From fatty acid (4)	Total (5)	Relative $C^{13}$ excess (6)	From fatty acid (7)	Total (8)	Relative $C^{13}$ excess (9)	From fatty acid (10)
	$\mu M$	per cent	$\mu M$	$\mu M$	per cent	$\mu M$	$\mu M$	per cent	$\mu M$
Octanoic	309	48	149	264	63	167	710	42	296
"	321	48	152	366	47	171	600	22	132
"	382	42	159				856	33	277
"							583	21	122
Hexanoic	295	38	113	525	36	189	955	36	344
"	513	39	200	507	40	202	622	20	124
"							1085	33	358
"							736	30	221
Butyric	348	32	113	472	37	173	496	24	119
"	390	31	122	616	40	249	525	16	84
Acetic	687	19	130	755	24	184	820	18	148
"	639	23	147	745	21	154	850	19	161
"	640	22	141	650	19	124			

tially equal distribution of  $C^{13}$  between the carboxyl and  $\beta$ -carbons, and no  $C^{13}$  in the  $\alpha$ - and  $\gamma$ -carbons. Further experiments with hexanoic and octanoic acids have confirmed these results. The accuracy of the thermal degradation procedure we employed was established by experiments with synthetic  $\beta$ - and carboxyl-tagged acetoacetate. Buchanan *et al.* (2) have found in similar experiments, however, a definitely higher  $C^{13}$  content in the carboxyl than in the  $\beta$ -carbon. Since the only obvious difference was their use of a chemical (aniline citrate) instead of thermal decarboxylation, we repeated several experiments, using the aniline citrate decarboxylation,

and we also observed an unequal distribution. In three experiments with COOH-tagged octanoate the isotope ratios for COOH- $\beta$ -carbon in the acetoacetates were 1.10, 1.37, and 1.52. It seems apparent that thermal decarboxylation yields  $\text{CO}_2$  from some source other than acetoacetate, and this source of error can be avoided by use of the more specific chemical decarboxylation. Inasmuch as we were interested only in the order of magnitude of the acetoacetate  $\text{C}^{13}$  excess, we have not attempted to correct our figures, but recognize that they are probably somewhat low.

TABLE II  
*Yields and  $\text{C}^{13}$  Excesses of Acetoacetate*

The same experiments listed in Table I.

Acid (1)	Fasted			Fed			Fasted + pyruvate 0.01 M		
	Total	Relative $\text{C}^{13}$ excess	From fatty acid	Total	Relative $\text{C}^{13}$ excess	From fatty acid	Total	Relative $\text{C}^{13}$ excess	From fatty acid
	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
	$\mu\text{M}$	per cent	$\mu\text{M}$	$\mu\text{M}$	per cent	$\mu\text{M}$	$\mu\text{M}$	per cent	$\mu\text{M}$
Octanoic	212	75	159	68	72	49	171	73	124
"	121	72	88	147	65	96	188	42	80
"	171	76	130				238	64	152
"							115	56	65
Hexanoic	150	55	83	149	58	86	199	49	98
"	219	63	139	135	55	75	162	*	*
"							116	35	41
"							85	48	41
Butyric	349	61	213	136	59	80	219	50	110
"	391	67	260	257	64	164	145	47	68
Acetic	166	33	54	39	26	10	90	25	23
"	123	38	47	24	*	*	85	23	20
"	130	36	47	41	23	9			

\* No determination.

In the special case of butyrate, from which acetoacetate can apparently be formed by direct oxidation as well as by fission and recombination (18), the value of 1.9 for the COOH- $\beta$ - $\text{C}^{13}$  ratio was used. This is the average of values previously observed and confirmed in subsequent experiments. To represent the relative  $\text{C}^{13}$  excess as the relative proportion of fatty acid carbon in the acetoacetate, it is necessary to assume that all of the fatty acid carbon contributes equally to the formation of acetoacetate. Although this assumption may not be strictly justified, particularly in view of the results of Buchanan *et al.* (2), it provides a useful approximation to the true values.

### Results

*Complete Oxidation of Fatty Acids by Liver of Fasted Rat*—Data on the yields and  $C^{13}$  excesses of the respiratory  $CO_2$  obtained in these experiments are summarized in Table I. Two observations are particularly noteworthy. First, despite a considerable degree of variability in the total respiratory  $CO_2$ , the relative  $C^{13}$  excesses are quite constant for each acid. With octanoate as substrate the fatty acid carbon makes up about half of the total respiratory  $CO_2$ , whereas with acetate only about 20 per cent of the  $CO_2$  was derived from the labeled acid. Apparently fatty acids of intermediate chain length tend to inhibit the oxidation of endogenous substrates. The significance of this finding is not clear and is now being studied further; it is important, however, in illustrating the variable effects different substrates may have on the endogenous metabolism and emphasizes the hazards involved in the common practice of calculating the rate of oxidation of a substrate by differences in oxygen consumption from endogenous levels.

The second observation is that the quantity of fatty acid carbon completely oxidized (Column 4) is of similar magnitude for all four acids, ranging from 113 to 200 microatoms per gm. of tissue per 2 hours. This suggests that liver tissue has a rather fixed capacity for oxidation of fatty acid carbon, regardless of the chain length of the acid.

*Comparison between Livers of Fed and Fasted Rats*—Data on respiratory  $CO_2$  from the fed rat livers, recorded in Column 6 of Table I, show that the presence of endogenous glycogen has no particular effect on the proportion of fatty acid carbon to endogenous carbon completely oxidized. With each acid, the relative  $C^{13}$  excess of the respiratory  $CO_2$  was of the same order in the "fed" livers as in the "fasted." In some experiments the rate of fatty acid oxidation was definitely higher in the "fed" than in the "fasted" liver. In these instances the increase was due to an elevation of the total respiratory activity of the tissue rather than to an increase in the proportion of fatty acid to endogenous carbon in the respiratory  $CO_2$ .

*Fatty Acid Oxidation in Presence of Pyruvate*—In view of the lack of effect of endogenous glycogen on the rates of oxidation of these fatty acids, it was of interest to test the effect of a carbohydrate intermediate which is known to be rapidly and completely oxidized by liver, namely pyruvic acid. It was expected that the presence of approximately equal molar concentrations of fatty acid and pyruvate would provide the best conditions for the demonstration of substrate competition. It is obvious, however, when the data of Column 10 are compared with those of Column 4, that pyruvate did not inhibit, but in many experiments enhanced the rate of fatty acid oxidation. Inasmuch as the presence of pyruvate raised the respiratory levels considerably in all experiments, it seems evident that,

aside from any accelerating effect pyruvate may have on fatty acid oxidation, the respective oxidations are additive rather than competitive.

*Acetoacetate Formation*—Although data on respiratory  $\text{CO}_2$  were of primary importance to this study, it is of interest to consider the effect of carbohydrate on acetoacetate formation in these experiments. These results, for the same experiments listed in Table I, are presented in Table II. Despite wide variations in total acetoacetate production (Column 2), the constancy of relative  $\text{C}^{13}$  excess for each acid was noted to be the same (Column 3). The proportion of fatty acid carbon in the acetoacetate ranged from 75 per cent for octanoate to 35 per cent for acetate, with intermediate values for hexanoate and butyrate. These levels, for each acid, are distinctly higher than those for the respiratory  $\text{CO}_2$ . In agreement with earlier experiments (9, 10) acetoacetate production was highest from butyrate and lowest from acetate (Column 4).

Comparison of Column 7 with Column 4 reveals that, despite some overlapping, acetoacetate formation was generally lower in the "fed" than in the "fasted" liver. This effect was most evident with butyrate and particularly with acetate as substrate. Because of the large individual variations among different experiments no definite conclusions can be drawn concerning the effect of pyruvate on acetoacetate formation. With butyrate and acetate as substrates its effect in lowering acetoacetate production is similar to that of the "fed" liver.

*Complete Oxidation versus Ketone Body Formation*—Of these two catabolic reactions of fatty acids occurring in liver slices, quantitatively ketone body formation is more significant, particularly under fasting conditions, where the conversion of fatty acid carbon to acetoacetate ranges up to 4 times the conversion to  $\text{CO}_2$ . The difference is even greater if we consider that acetoacetate is always accompanied by one-fourth to one-half its amount of  $\beta$ -hydroxybutyrate. Comparison with data on "fed" liver suggests that where an effect of carbohydrate is evident it is to increase the conversion to  $\text{CO}_2$  and to decrease the conversion to ketone bodies.

Though no attempt was made in these experiments to account quantitatively for all of the fatty acid which disappeared, conversion to  $\text{CO}_2$  and to acetoacetate accounted for from 50 to 80 per cent; a substantial part of the remainder must have been converted to hydroxybutyrate, which was determined in only a few experiments. Isotopic analysis of the residual solutions and tissues revealed no significant accumulation of  $\text{C}^{13}$ , though its presence in small amounts could have been masked by the large quantities of normal carbon.

*Effects of Other Substances on Octanoate Oxidation*—The effects of several other substances on the oxidation of octanoate by fasted rat liver slices are given in Table III. Glycerol and glucose were tried, because these were



found by Edson (8) to be strongly antiketogenic. No significant differences were observed, however; the yields of  $\text{CO}_2$  and acetoacetate, and the proportions of fatty acid carbon therein, were of the same order as with octanoate alone.

Malonate in 0.02 M concentration inhibited total respiration by about two-thirds, but almost completely blocked the oxidation of octanoate. Acetoacetate production was completely inhibited. The addition of fumarate restored the oxidation of octanoate slightly, but did not restore acetoacetate production. This somewhat surprising observation suggests that malonate exerts some effect other than inhibition of succinate oxidation (19).

TABLE III

*Effect of Easily Oxidizable Substrates on Octanoate Oxidation by Fasted Rat Liver Slices*

Conditions same as in Table I.

Additions	Respiratory $\text{CO}_2$			Acetoacetate		
	Total	Relative $\text{C}^{18}$ excess	From fatty acid	Total	Relative $\text{C}^{18}$ excess	From fatty acid
	$\mu\text{M}$	per cent	$\mu\text{M}$	$\mu\text{M}$	per cent	$\mu\text{M}$
Glycerol, 0.01 M . . . . .	303	49	149	133	83	111
Fumarate, 0.01 M . . . . .	450	42	191	172	74	127
Glucose, 0.01 " . . . . .	445	46	205	153	81	124
" 0.05 " . . . . .	550	43	238	146	73	106
Malonate, 0.02 " . . . . .	168	2	3	0		
Same + fumarate, 0.01 M . . . .	215	8	17	0		

This effect of malonate, which we have confirmed in numerous experiments with non-isotopic fatty acids in slices and in washed and crude homogenates, is in marked contrast to the results of Lehninger (3) who found that conversion of octanoate to acetoacetate was only slightly sensitive to the presence of malonate. The only explanation is a difference in strain of rats (*cf.* Lehninger (3)).

#### DISCUSSION

Inasmuch as the present study has provided no support for the idea that carbohydrate is oxidized preferentially to fatty acids in liver, it is of interest to consider other possible explanations for the antiketogenic action of carbohydrate. A considerable body of evidence has accumulated to indicate that carbohydrate may exert its effect at the 2 carbon level of fatty acid breakdown (20, 21). By supplying oxalacetate (presumably by carboxylation of pyruvate) carbohydrate can direct the metabolism of the acetyl

derivative formed by  $\beta$  oxidation of fatty acids to complete oxidation via the Krebs cycle. In the absence of oxalacetate, an accumulation of acetyl groups would lead to their self-condensation to acetoacetate. Not only is this theory in full accord with the results of these experiments, but it affords a reasonable explanation for the apparent increase in the rate of oxidation of these fatty acids brought about by endogenous glycogen or added pyruvate. It also supplies an explanation for the findings of Bobbitt and Deuel (22) that glycogen added to liver slices increased the rate of butyrate utilization but decreased the rate of ketogenesis, a finding which they attributed to "ketolysis."

The possibility that carbohydrate exerts a "ketolytic" effect by catalyzing the further oxidation of ketone bodies remains for consideration. This hypothesis is now under investigation.

The authors express their appreciation to the Sun Oil Company, Philadelphia, for its support and interest and to Mr. Arthur Kent for the  $C^{14}$  analyses.

#### SUMMARY

The theory that carbohydrate inhibits ketogenesis by competitively inhibiting fatty acid oxidation was tested by measuring the effect of endogenous glycogen and added pyruvate on the rates of oxidation of a series of short chain, isotopically labeled fatty acids, the extent of oxidation being determined by the amount and isotopic content of the respiratory  $CO_2$ . Not only was there no inhibition, but in many experiments carbohydrate increased the rate of fatty acid oxidation.

Ketogenesis was inhibited in the "fed" liver and by the addition of pyruvate when acetate and butyrate were used as substrates, but was essentially unchanged when hexanoate and octanoate were used.

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# STUDIES ON OVOMUCOID\*

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Recent correlation by Lineweaver and Murray (1) of the antitryptic factor of chicken egg white with ovomucoid, together with further studies by Lineweaver *et al.* (2, 3) on its interaction with trypsin, have stimulated interest in this protein. From its resistance to heat and to the precipitating action of various reagents, ovomucoid has been considered to be a very stable protein, although no evidence has been presented that such treatments do not alter its structure. The methods used to prepare it (4) involve conditions sufficiently drastic to denature many other proteins. The purpose of this investigation was to develop a method for the separation of ovomucoid under somewhat less drastic conditions and, furthermore, to examine the physical and chemical properties. The relatively high solubility of ovomucoid in trichloroacetate systems, previously recognized (1, 5), has in part formed the basis of the fractionation methods.

## EXPERIMENTAL

The ovomucoid was prepared from fresh egg white by using a combination of sodium trichloroacetate precipitation with ethanol fractionation. The various ovomucoid preparations used in the studies on chemical characterization were thoroughly dialyzed against distilled water at the isoelectric point, lyophilized, and then further dried in a vacuum oven at 60° for 24 hours.

Electrophoretic analyses of ovomucoid fractions were carried out in a sodium diethyl barbiturate buffer of ionic strength 0.1 at pH 8.6. Mobility experiments were carried out in buffers of 0.1 ionic strength, sodium chloride constituting 80 per cent of the salt. In the experiments at ionic strength 0.01, buffer salt alone was used.

Velocity sedimentation analyses were performed in the Svedberg oil turbine ultracentrifuge, a schlieren optical system being used to record the boundaries.<sup>1</sup>

Diffusion experiments were carried out in an electrophoretic apparatus

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† Fellow of the Belgian-American Educational Foundation, 1948-49.

<sup>1</sup> The sedimentation experiments were carried out by Mr. E. M. Hanson.

at 1.2° with the conventional schlieren lens system. The boundary sharpening technique of Kahn and Polson (6) was utilized. The value for the diffusion constant was corrected to 20° in the usual manner (7).

Heterogeneity constants were determined by the method of Alberty (8). The electrical spreading was determined at a potential gradient of 1.7 volts per cm.

Viscosity determinations were performed in an Ostwald viscometer at 25°, in a solvent 0.1 M in sodium chloride and 0.05 M in sodium acetate buffer at pH 3.9. In studies of the effects of 8 M urea on ovomucoid the solutions were allowed to stand at least 1 hour before viscosity measurements were made.

The partial specific volume of the protein was determined in water at 25° in a pycnometer of approximately 20 ml. volume.

The well known tryptic inhibitor activity of ovomucoid was determined by the procedure of Lineweaver and Murray (1) and was referred to the activity of dry egg white prepared by lyophilization. Tryptic activity was measured by the hemoglobin method of Anson (9).

Nitrogen determinations were carried out by a micro-Kjeldahl procedure.

The carbohydrate content of the protein was measured by the orcinol method of Sørensen and Haugaard (10) and the carbazole method of Dische (11), with mannose as the reference standard. Glucosamine was determined by Hewitt's (12) method on ovomucoid samples hydrolyzed 8 hours at 100° in 2 N HCl. Extinctions were measured with a Beckman spectrophotometer.

### *Results*

The method utilized for the preparation of ovomucoid depends in part upon its relatively high solubility in sodium trichloroacetate solutions. The most suitable conditions of pH and trichloroacetate concentration for the removal of non-ovomucoid proteins were studied with whole egg white at 1:6 dilutions with distilled water. Picrate ion likewise gives satisfactory results in these studies at even lower concentrations than trichloroacetate ion. The other egg white proteins are relatively insoluble in the presence of the above anions at pH values acid to their isoelectric points. The main contaminant of the ovomucoid in the supernatant solution under such conditions is ovalbumin. The solutions, after the initial precipitation carried out with trichloroacetate ion, were dialyzed at pH 3.9 to remove the salt; the protein was then recovered by lyophilization. In Table I are shown the data for yield and purity of ovomucoid as a function of pH and trichloroacetate ion concentration.

The ovomucoid so obtained consists of two closely related electrophoretic components and will be referred to as "crude" ovomucoid (Precipitate II,

Diagram 1). The minor component made up approximately 5 to 8 per cent of the total. In buffers at pH 8.6 and ionic strength 0.1, it migrates

TABLE I  
*Conditions for Precipitation of Non-Ovomucoid Proteins of Whole Egg White by Trichloroacetate*

Precipitating conditions		Composition of supernatant solution		
pH	Trichloroacetate concentration	Protein per liter egg white	Ovomucoid	Ovalbumin
	<i>per cent</i>	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
4.9	3	72	17.5	75.5
4.0	3		38.0	55
4.0	5	22	72	24
3.5	5	14	99-100	0-1
2.8	3	17	95	4

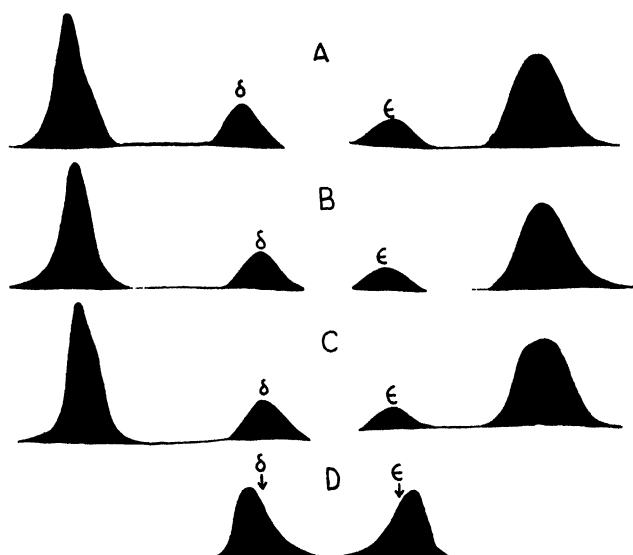


FIG. 1. Electrophoretic patterns of ovomucoid fractions at ionic strength 0.1. Duration of Experiments A, B, and C, 10,800 seconds at a potential gradient of 6 volts per cm. A, crude ovomucoid at pH 8.6 (Precipitate II, Diagram 1); B, purified ovomucoid at pH 8.6 (Precipitate II-C, Diagram 1); C, purified ovomucoid residue at pH 8.6 (Precipitate II-B, Diagram 1); D, purified ovomucoid at pH 4.1 (8100 seconds, potential gradient 3.7 volts per cm.).

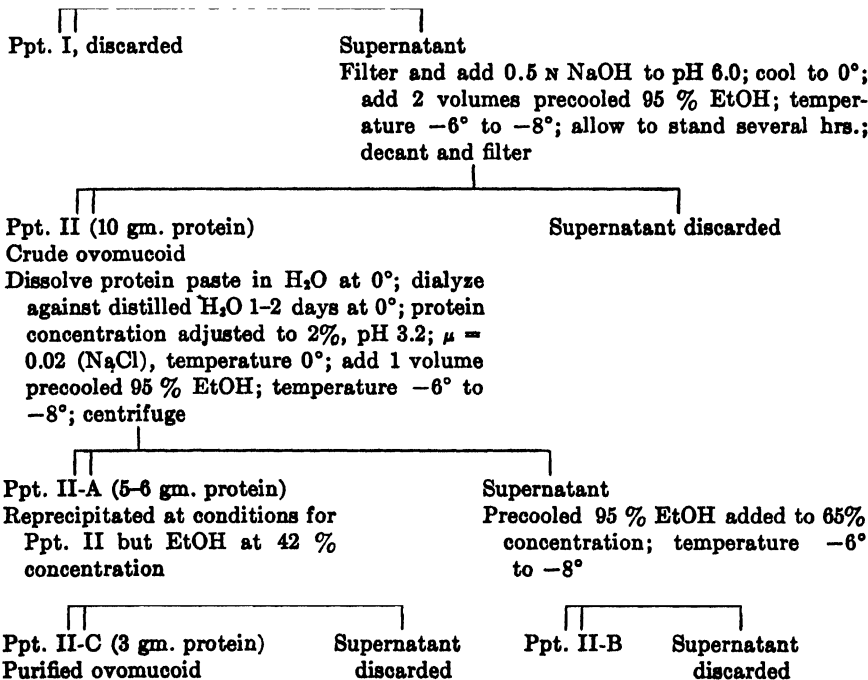
as a slow and poorly resolved shoulder. An ovomucoid fraction which is free of this minor component was prepared by ethanol precipitation. Conditions for its separation as well as the preliminary fractionation steps are

shown in Diagram 1. To avoid the use of excessive volumes and reagents, all fractionations were carried out on undiluted egg white. The success of the several separations is indicated by the electrophoretic patterns in Fig. 1. The material which has given rise to Fig. 1, *B* will be designated hereafter as "purified" ovomucoid (Precipitate II-C, Diagram 1). This

#### DIAGRAM 1

##### *Preparation of Ovomucoid*

1 liter fresh egg white dispersed with Waring blender;  
adjust pH to 3.5 with 1 *N* H<sub>2</sub>SO<sub>4</sub>; add 1 volume 10%  
sodium trichloroacetate, pH 3.0; final pH adjusted  
to 3.5 (25°); allow to stand overnight; decant and  
centrifuge



protein was extensively characterized. As indicated by Diagram 1, this substance represents the less soluble portion of the crude ovomucoid. The more soluble portion (Precipitate II-B, Diagram 1, and Fig. 1, *C*) shows an enhanced amount of the more slowly migrating component.

##### *Electrophoretic Studies*

The so called purified ovomucoid showed a single boundary upon electrophoretic study in buffers of 0.1 ionic strength, although, as is evident

in Fig. 1, *D*, there is some asymmetry near the isoelectric point. A plot of electrophoretic mobilities *versus* pH (Fig. 2) gives an isoelectric point of 3.9. This value is somewhat lower than that reported by Longworth, Cannan, and MacInnes (13) and by Hesselvik (14) for ovomucoid prepared by differential heat denaturation of the other egg white proteins.

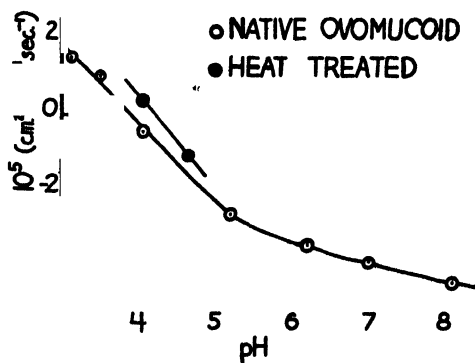


FIG. 2. pH-mobility curve of ovomucoid at ionic strength 0.1

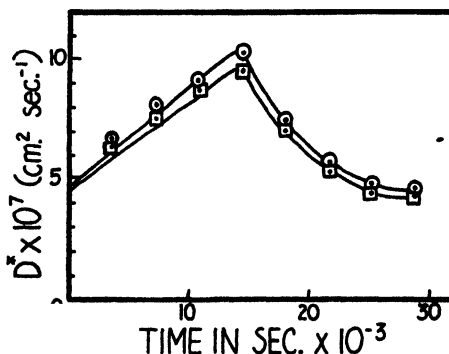


FIG. 3. Variation of the apparent diffusion constants ( $D^*$ ) of ovomucoid during electrophoresis at ionic strength 0.1 and pH 3.9. Current reversed after 14,400 seconds.  $\square$  and  $\circ$  represent the experimental data for the right and the left limbs of the cell.

Heating purified ovomucoid for 1 hour at  $100^\circ$  at pH 3.9 in low salt concentration does shift the isoelectric point to pH 4.2, which is in good agreement with the value of 4.3 found by Longworth, Cannan, and MacInnes, but lower than Hesselvik's value of 4.5.

The electrophoretic inhomogeneity of ovomucoid prepared by heat treatment has been previously recognized by Longworth, Cannan, and MacInnes (13). Our purified preparations were studied quantitatively in this respect by the method of Alberty (8). In Fig. 3, the apparent



diffusion constants at  $1.5^\circ$  in both cell limbs, obtained in buffer of pH 3.9, ionic strength 0.1, at a potential gradient of 1.7 volts per cm., are plotted against time in seconds. The curve maxima indicate the point at which the current was reversed at 14,400 seconds. After complete electrophoretic reversal, the apparent diffusion constant was found to equal the diffusion constant obtained by the usual procedure. Since the boundary spreading was completely reversible, a heterogeneity constant was calculated. Values of  $0.51 \times 10^{-5}$  and  $0.55 \times 10^{-5}$  were found for the two limbs. Curves constructed from these values agree reasonably well with the experimental

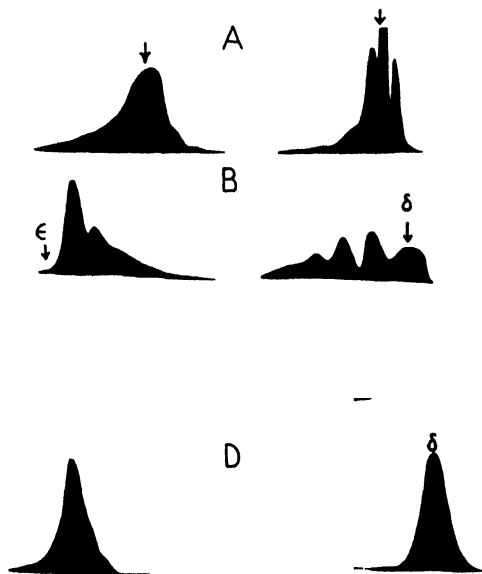


FIG. 4. Electrophoretic patterns of purified ovomucoid at ionic strength 0.01. Duration of experiments 10,800 seconds. *A*, *C*, and *D* 2.1 per cent protein; *B*, 1.1 per cent; *A*, pH 4.0; *B*, pH 4.5; *C*, descending boundary at pH 8.6; *D*, ascending boundary at pH 8.6. Arrows denote the positions of the initial boundary.

points, except during the early stages of the experiment, when the errors in the determination of the apparent diffusion constant are relatively large.

The mean heterogeneity constant of  $0.53 \times 10^{-5}$  is of the same order of magnitude as that found for some other protein systems (15, 16). By the use of this constant and the value of  $2 \times 10^{-5}$  for the slope of the mobility-pH curve of Fig. 2, the standard deviation for the distribution of the isoelectric point of ovomucoid is calculated to be 0.26 unit.

Electrophoretic patterns of purified ovomucoid in buffers of ionic strength 0.01 and different pH values are shown in Fig. 4. These experi-

ments were also carried out at a constant potential gradient of approximately 6 volts per cm. A resolution which is particularly evident on the ascending limb indicates at least five electrophoretic maxima at pH 4.5, as shown by Fig. 4. The effects noted are electrophoretically reversible. Resolution of these maxima is less complete upon electrophoresis at pH 4.0, and, at pH 8.6, ovomucoid shows only a pronounced asymmetrical broadening on the descending limb. It has been previously pointed out by Alberty (8) that heterogeneities in protein systems are more readily discerned at the lower ionic strengths. In any event, the electrophoretic experiments with ovomucoid at ionic strength 0.01 indicate the presence of at least two components in the system, whereas at ionic strength 0.1 only one is indicated. Heating ovomucoid preparations at 100° for 1 hour

TABLE II  
*Sedimentation Experiments on Ovomucoid Preparations*

Material	Protein concentration	Sedimentation constant $s_{20,w}$
	per cent	Svedberg units
Electrophoretically separated ovomucoid	0.4	2.7
	1.2	2.8
Crude ovomucoid, alcohol precipitation	0.9	2.8
“ “ ammonium sulfate precipitation	0.75	2.9
Purified ovomucoid, pH 3.9	0.5	2.75
“ “ “ 3.9	0.9	2.85
“ “ “ 3.9	1.5	2.7
“ “ “ 1.4	0.75	2.75
“ “ “ 11.6	0.8	2.7
Treated by 5% trichloroacetic acid	0.5	2.8
Heat-treated	0.7	2.6

does not change their electrophoretic properties in diethyl barbiturate buffer of pH 8.6 and ionic strength 0.1.

#### *Sedimentation Studies*

The sedimentation constants of various ovomucoid preparations were determined under a variety of conditions. These results are presented in Table II. Ovomucoid shows a single symmetrical boundary, which indicates that in the systems considered the protein is substantially homogeneous. No difference in the sedimentation constant of ovomucoid prepared by fractionation methods and by electrophoretic isolation was apparent. The sedimentation constant was independent of protein concentration over a 0.5 to 1.5 per cent range and gave an average  $s_{20,w}$  = 2.8 Svedberg units. Crude ovomucoid gave the same values as did the

purified material. No difference in ovomucoid precipitated from the trichloroacetate solutions by ethanol and ammonium sulfate was apparent. Moreover, ovomucoid was not affected by previous treatment with 5 per cent trichloroacetic acid. Sedimentation experiments at pH 1.4 to 11.6 likewise revealed no change. Ovomucoid solutions heated for 1 hour at  $100^{\circ}$  consistently gave a slightly lower sedimentation constant ( $s_{20w} = 2.6$  Svedberg units) than did unheated material. A sedimentation experiment at very low salt concentration at pH 3.9 showed a single boundary. The sedimentation diagram of purified ovomucoid in 0.15 M NaCl at pH 3.9 is shown in Fig. 5.

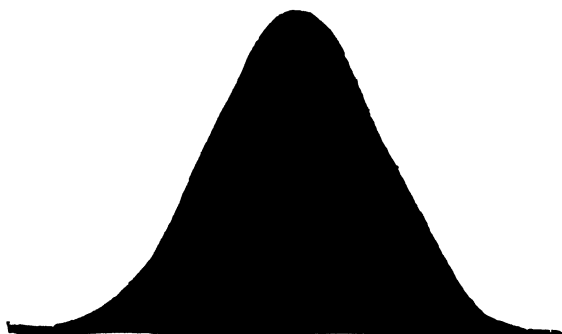


FIG. 5. Sedimentation pattern of purified ovomucoid after 160 minutes at  $185,000 \times g$ .

#### *Diffusion Constants*

Values for the diffusion constant at  $1.2^{\circ}$  at protein concentrations of 0.6 and 0.9 per cent were found to be  $4.45$  and  $4.25 \times 10^{-7}$  cm.<sup>2</sup> sec.<sup>-1</sup> respectively. The mean value of these determinations, when corrected to  $20^{\circ}$ , is  $8.0 \times 10^{-7}$  cm.<sup>2</sup> sec.<sup>-1</sup>.

#### *Apparent Specific Volumes*

This constant ( $V_{sp}$ ) was determined at protein concentrations of 1.60, 1.09, and 0.79 per cent respectively. The corresponding  $V_{sp}$  values were 0.684, 0.686, and 0.679. Since the last value was subject to greater errors because of the lower protein concentration, the mean of the first two values, 0.685, was used. This figure is considerably lower than the  $V_{sp}$  observed for most proteins (7), although Carter has reported 0.658 for thymus nucleohistone (17). From the values for the sedimentation and diffusion constants and the partial specific volume, a molecular weight of 27,000 is calculated. This is in agreement with the value of 28,800

reported by Lineweaver and Murray (1), obtained from osmotic pressure measurements on ovomucoid.

### Viscosity Studies

The specific viscosity of ovomucoid was found to be proportional to the concentration within the limits of experimental error. As is shown in Fig. 6, heat-treated ovomucoid ( $100^\circ$  for 1 hour) showed only a very slight increase in viscosity. It is interesting that material treated in identical fashion gave a slightly lower sedimentation constant, a change which might be due to the increased viscosity. Treatment with 8 M urea did, however, cause a small increase in viscosity. From the viscosity increment of 8, an axial ratio of 6.5, calculated as a prolate ellipsoid, is

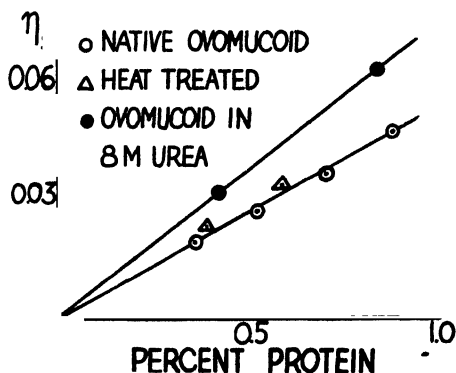


FIG. 6. Effect of heat treatment and 8 M urea on the specific viscosity of ovomucoid.

obtained from the tables of Mehl, Oncley, and Simha (18). From the sedimentation and diffusion constants a value of 1.35 is obtained for the frictional ratio. This gives an axial ratio of 6.3, which is in good agreement with the value obtained by viscosity studies. Since the degree of hydration is not known, the figures obtained are necessarily maximum values.

### Other Studies

Purified ovomucoid was found to contain 13.2 per cent nitrogen, in agreement with the results of Lineweaver and Murray (1). The ash content was less than 0.05 per cent. No trichloroacetate ion was bound by the purified ovomucoid. The sample showed 9.7 per cent mannose as determined by the orcinol method. The carbazole method of Dische (11) gave a carbohydrate value of 9 per cent when the extinction at  $540\text{ m}\mu$  was compared with that of pure mannose.<sup>2</sup> These results are in fair

<sup>2</sup> We wish to thank Miss Margaret E. Marshall for carrying out this determination.

agreement with those reported by Sørensen (19). An average of four determinations gave a glucosamine value of 17 per cent, which is somewhat higher than that reported by most workers (4). The nitrogen, glucosamine, and mannose content of the various ovomucoid preparations of Fig. 1 showed no apparent differences.

The antitryptic activities of the various ovomucoid fractions were essentially identical. The samples gave a value of 8 antitryptic units per mg. of protein, compared to the value of 1 unit per mg. of dried egg white as defined by Lineweaver and Murray (1). Our results are quite consistent with these values. Failure to discern any difference in activity between the various fractions assayed may well be due to the limitations of the method used. Attempts to crystallize ovomucoid from concentrated ammonium sulfate solutions were unsuccessful.

#### DISCUSSION

Ovomucoid is a mucoprotein of unusual stability. The viscosity data indicate that no denaturation is apparent after the protein is heated for 1 hour at 100°. However, biological inactivation in the form of loss of antitryptic activity has been shown by Lineweaver and Murray (1) to occur under such conditions. The shift in the isoelectric point likewise shows that undefined changes have occurred. This emphasizes the danger of using heating procedures to prepare mucoproteins, despite their unusual stability. In 8 M urea solution, only small changes appear in specific viscosity.

At ionic strength 0.01 and pH 4.5, ovomucoid shows the presence of at least five electrophoretic maxima. In sedimentation analysis under the same conditions there is a single boundary. Thus it would appear that the electrophoretic results are not due to a dissociation of this protein into smaller molecular fragments. Our present knowledge of electrophoretic phenomena occurring at low ionic strength does not permit an explanation of these results. A somewhat similar situation has been previously noted by Sharp *et al.* (20) for horse serum albumin. The possibility of anomalies arising from the binding of buffer ions may be a factor in the apparent complexity of the electrophoretic behavior in this system.

If the heat stability of ovomucoid is due to its high carbohydrate content, it would appear that the various protein molecules present are mucoproteins, since the system showed such extreme resistance to denaturation. The high density of this protein (proteins) may be related to its carbohydrate content, which, in terms of mannose and glucosamine, constitutes approximately 27 per cent of ovomucoid.

The electrophoretic behavior in buffers of ionic strength 0.1, while

showing ovomucoid to be relatively heterogeneous, does not disclose the complexity seen at ionic strength 0.01. In order to evaluate this property of ovomucoid more fully, extensive fractionation of this system will be required. It might be well to point out the inconsistency of interpreting the results of fractionation experiments carried out at low ionic strengths in terms of electrophoretic analyses at ionic strength 0.1. Thus, upon electrophoresis at the latter ionic strength, one is led to the conclusion that a fairly homogeneous protein has been separated, while great complexity may be indicated in buffers of ionic strength 0.01. Since the sub-fractionation used in preparing so called purified ovomucoid was carried out at an ionic strength of approximately 0.01, one might suggest that in terms of the electrophoretic diagram shown in Fig. 4, *B* the possibilities for further separation are indicated.

#### SUMMARY

Ovomucoid has been prepared under conditions of fractionation which are less rigorous than those usually employed, giving a substance which is substantially monodisperse. A combination of data on sedimentation, diffusion, viscosity, and partial specific volume for this very stable mucoprotein gives a molecular weight of 27,000 and an axial ratio of approximately 6.4.

In buffers of ionic strength 0.1 the ovomucoid behaves as a single electrophoretic component with a heterogeneity constant of  $0.53 \times 10^{-5} \text{ cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ .

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# A RAPID COLORIMETRIC METHOD FOR THE DETERMINATION OF SODIUM IN BIOLOGICAL FLUIDS AND PARTICULARLY IN SERUM

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Despite the many procedures and their modifications (1-4) which have appeared in the literature, there is still need for a more satisfactory method for determining sodium in serum. The normal range of serum sodium (310 to 330 mg. per cent) has a percentage variation which is considerably less than that of any other blood constituent commonly estimated. Thus it is evident that an adequate method for the determination of sodium in serum should be accurate to considerably better than 5 per cent, a criterion which very few of the available methods can meet.

The flame photometer, while extremely sensitive and capable of a high order of accuracy, has not met with great success because of the technical difficulties involved in its use (5). The apparatus is expensive and often conflicts with local fire ordinances when propane is used for combustion. A sensitive nephelometric method has been described by Lindsay, Braithwaite, and D'Amico (1), but it has not achieved great popularity. The older, purely chemical methods seem in general to be the most reliable. Basically, they all depend on the formation of a somewhat insoluble salt of sodium-uranium-zinc or sodium-uranium-magnesium acetates. The best of these methods require preliminary ashing of the serum, a final gravimetric step, or both, and these procedures are inconvenient and time-consuming. Attempts to circumvent the difficulties by colorimetric modifications have been hampered by the inherently low color intensity of aqueous solutions of the triple salt. Barrenscheen and Messiner (6) attempted to intensify the color of the triple salt by the addition of potassium ferrocyanide, but the reaction has been found to be far too sensitive to variations in temperature and reagent concentration. In fact, the color of the triple salt solution itself is affected by temperature changes. Hoffman and Osgood (7) found that the addition of ammonium thiocyanate to solutions of the triple salt lessened the temperature effect but did not increase the color intensity.

During the course of an investigation of the interdependence of sodium and chloride excretion (8), it was found necessary to develop a simple and more accurate method for the determination of sodium in biological fluids



than was presently available. In a preliminary report (9) we outlined a rapid colorimetric procedure which was suitable for the determination of sodium in serum and urine by the average hospital, and which did not require any special apparatus or techniques. The method was based on the Rosenheim-Daehr reaction (10) in which the uranyl ion, in strongly alkaline solution, is treated with hydrogen peroxide to produce a complex of intensely reddish yellow color. Arnold and Pray (11) had studied this reaction in the development of a sodium method but had made no attempt to adapt it to biological uses.

It was realized at the time our method was developed that, inasmuch as the triple salt was somewhat soluble, the conditions of the procedure would have to be firmly fixed in order to obtain reproducibility of results. With continual use it was found that it would be advantageous to change some of the set conditions of the method reported in our previous paper (9). For example, the precipitate of the triple salt was too voluminous for convenient handling. In view of the special problem presented by serum sodium, it was decided to attempt to increase the accuracy of the method. At the same time a more detailed investigation was made of the spectrophotometric characteristics of the uranium complex, the influence of temperature on the reaction, the selection of optimum wave-length for measurement, and the accuracy, reproducibility, and recovery obtained by the method.

#### EXPERIMENTAL

##### *Reagents—*

1. *Uranyl zinc acetate.* A solution of 14 ml. of glacial acetic acid in 750 ml. of distilled water is brought almost to a boil. 77 gm. of uranyl acetate are added. To this mixture 231 gm. of zinc acetate, divided into five or six portions, are added with frequent stirring. Finally, 7 ml. of glacial acetic acid are added, and the solution is cooled to room temperature and diluted to 1 liter. Thereafter, 200 ml. of 95 per cent ethanol are added, and the solution is refrigerated overnight and filtered.

2. *Triple salt.* To 10 or 15 ml. of the above solution there is added enough of a concentrated sodium chloride solution to remove all but a trace of yellow color from the supernatant liquid. The precipitate is collected by filtration and washed five times with glacial acetic acid and five times with ether, then dried.

3. *Wash reagent.* A solution of 425 ml. of 95 per cent ethanol and 75 ml. of glacial acetic acid is saturated at room temperature with pure triple salt (see above). The reagent is stored in a brown bottle.

4. *Ammonium carbonate.* A saturated aqueous solution.

5. *Hydrogen peroxide,* 30 per cent (superoxol).

6. *Trichloroacetic acid.* A 10 per cent aqueous solution.

### *Apparatus*

The Beckman spectrophotometer, model DU, and the Leitz Rouy photometer were used for color measurements.

### *Method*

*Protein-Free Filtrate*—To 9.0 ml. of 10 per cent trichloroacetic acid is added 1.0 ml. of serum dropwise. The mixture is shaken or stirred thoroughly and centrifuged.

*Precipitation of Triple Salt*—1.0 ml. of the protein-free filtrate is pipetted into a 15 ml. graduated centrifuge tube and 6.0 ml. of the uranyl zinc acetate reagent are added. The solutions are mixed well by holding the tip of the centrifuge tube between the thumb and forefinger and rotating it rapidly back and forth. The tube is allowed to stand for 20 minutes, during which it is twirled twice by the same maneuver in order to stir up the precipitate. The tube is then centrifuged at high speed for 7 minutes, the supernatant liquid is decanted, and the tube allowed to drain for 1 minute.

*Washing Precipitated Triple Salt*—5 ml. of the wash reagent are added, care being taken to wash down the sides of the tube as well. The contents of the tube are mixed by twirling, and it is then centrifuged for 7 minutes, decanted, and drained.

*Color Development*—The precipitate is dissolved in a few drops of distilled water and 6 ml. of ammonium carbonate are added. Then, 1 ml. of 30 per cent hydrogen peroxide is added, and the volume is adjusted to 15.0 ml. with distilled water. After mixing by inversion, the solution is ready for reading in the colorimeter. When handling substantial numbers of samples, it is wise to add the peroxide and dilute to volume only two to three tubes at a time, in order to forestall the formation of gas bubbles which may interfere with accurate colorimetric readings.

*Measurement*—The sample is read in a photoelectric colorimeter with a 460  $m\mu$  filter.

### *Calibration*

Calibration curves may be prepared in two ways: either by serial dilution of a standard solution of the triple salt and subsequent color development, or by running known sodium (chloride) solutions through the entire procedure. It was of obvious importance to compare the agreement of these two techniques, as it would give an indication of the magnitude of technical errors involved in the preparation of the precipitate. Fig. 1 shows curves obtained with both methods. It appears that there is a small but constant loss of sodium involved in the preparation of the precipitate from the standard sodium solution, for serial dilutions of the triple

salt yield slightly more intense colors throughout. This loss evidently represents the sum of the mechanical losses (which are probably insignificant) and the loss due to the slight solubility of the precipitate, which is probably the more important factor. Although a small, empirical correction factor could be calculated from these curves and the calibration of the instrument simplified by the use of triple salt solutions, it appears wiser to us to calibrate by the use of sodium (chloride) solutions run

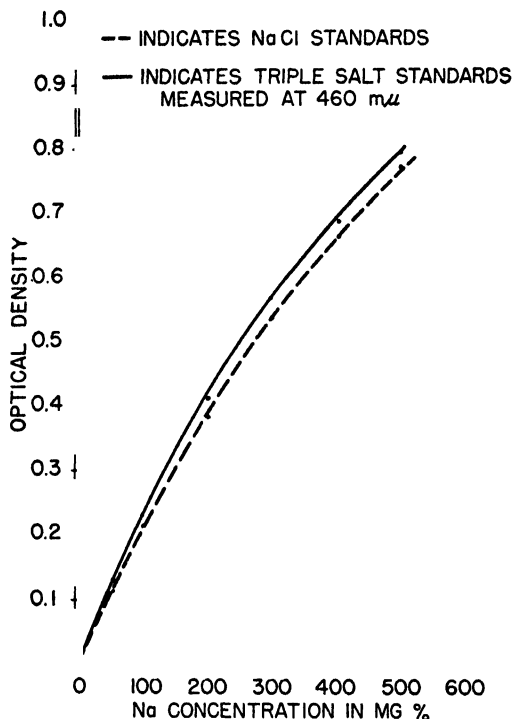


Fig. 1. Calibration curves prepared with NaCl and triple salt solutions

through the entire procedure. In this way, the calibration is adjusted to the technique of the individual operator, and results are more likely to approximate absolute values than when empirical corrections are introduced.

#### *Filters*

With use of the Beckman spectrophotometer, the absorption spectra of various concentrations of the final colored product were determined, as shown in Fig. 2. The peak absorption occurs just below 400 mμ, and there the variations with concentration are greatest. Theoretically, measurements carried out at or near this wave-length should yield the best results,

but in actual practice this was found not to be the case. The reasons for this discrepancy are evident from Fig. 3. Here are plotted the curves obtained from a series of standard sodium (chloride) solutions developed in the manner described and measured with the filters available in our colorimeter (the Leitz Rouy photometer). With use of the 415  $m\mu$  filter (the closest available to the theoretical 400  $m\mu$ ), a curve was obtained which indicates

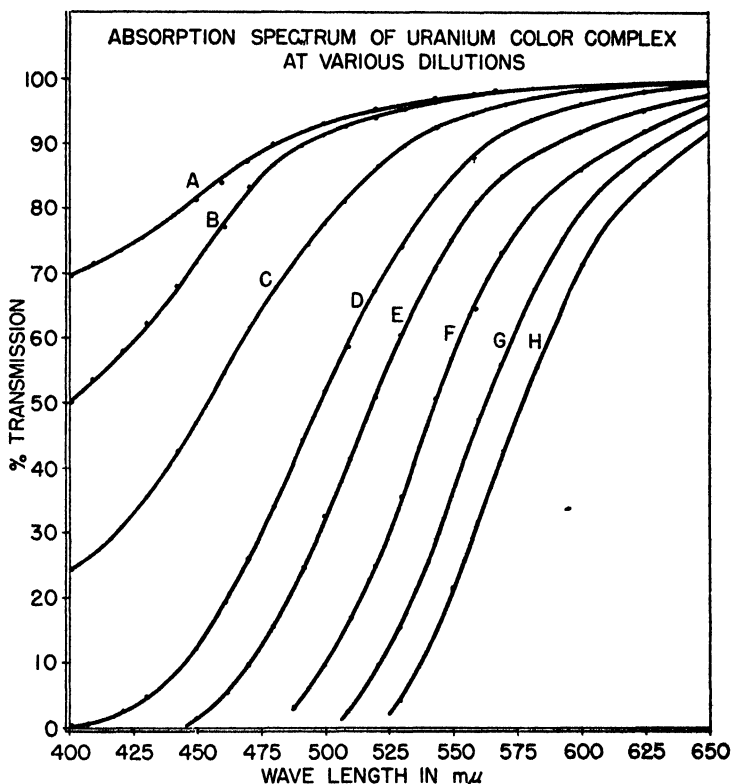


FIG. 2. Curves A through H represent sodium concentrations of 0.02, 0.08, 0.1, 0.3, 0.5, 1.0, 2.0, and 5.0 mg. per ml. of final solution, respectively.

that Beer's law is obeyed, but the concentrations of most interest, namely the normal serum sodium values of 310 to 330 mg. per cent, fall in the range of poor instrumental sensitivity in which large errors are involved.

To avoid this, and to bring the readings for normal values into the range of maximum instrument sensitivity, either a smaller sample could be used to give less intense colors, or another filter could be employed. The size of the sample was of necessity fixed so as to yield the optimum amount of precipitated triple salt. Lessening that amount would entail an in-

creasingly large and variable experimental error, inasmuch as the triple salt is by no means completely insoluble and the loss by incomplete precipitation and by washing of small quantities of precipitate would be proportionately great. It was evident, therefore, that other filters would have to be considered.

At 520  $m\mu$ , Beer's law is followed fairly well, but the sensitivity is again very low and the precision therefore is not good. The use of filters with transmission peaks at 445 and 460  $m\mu$  resulted in deviations from Beer's

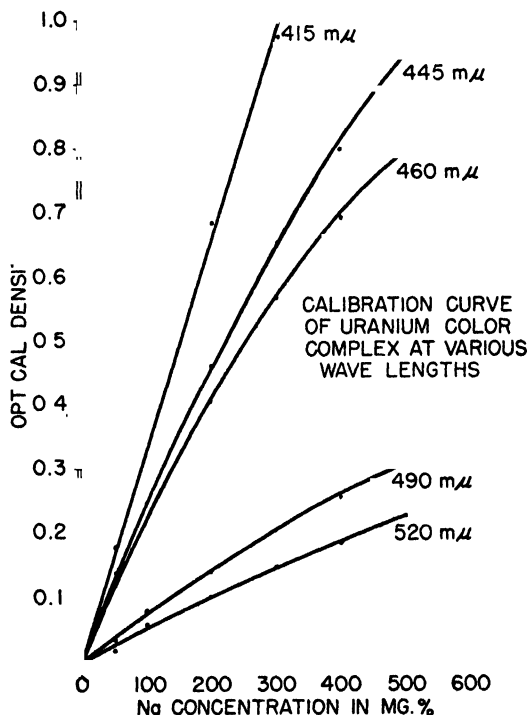


FIG. 3

law, but the precision in the normal sodium range was far better. Inasmuch as the sodium content of serum falls within such a narrow range of concentrations, the precision becomes more important than obedience to Beer's law. The 460  $m\mu$  filter was therefore chosen for the method and used for all further measurements.

It should be emphasized at this point that the various makes of filter photometers and spectrophotometers have different characteristics. When the method is to be used with instruments other than the Leitz Rouy photometer, it is advisable to determine which of the available filters gives maximum sensitivity in the concentration range to be studied.

*Stability of Complex Ion*

Numerous observations have been made at various concentrations over a period of 2 hours, and no detectable change in the color intensity was observed. After a variable period of time, however, there is a tendency for the formation of bubbles along the walls of the cuvettes. We have found it advisable to interrupt the simultaneous processing of a large number of specimens after the addition of ammonium carbonate. The addition of peroxide, dilution to final volume, and reading in the colorimeter are carried out on a small number of tubes at a time. This shortens the time between the addition of peroxide and final reading and eliminates the bubble nuisance without increasing appreciably the time required for the manipulation of a large number of samples.

TABLE I  
*Effect of Temperature on Precipitation of Triple Salt*

\* The figures represent actual meter readings.

Na concentration	20°	25°	30°
<i>mg. per cent</i>			
250	33.1	33.2	33.0
	32.5		
300	28.0	28.8	29.1
	28.9		
350	22.9	22.4	24.0
	23.0		

*Effect of Temperature*

In older methods employing the unaltered solutions of the triple salt, difficulties were sometimes encountered because of the influence of temperature. It was necessary, therefore, to investigate the effect of temperature both on the precipitation reaction and on the final color complex.

The precipitation reaction was studied by preparing 1:10 dilutions of standard solutions of sodium chloride equivalent to 250, 300, and 350 mg. per cent of sodium respectively. 1 ml. samples of each of these solutions were precipitated in the usual manner at temperatures of 20°, 25°, and 30°. The precipitates were then treated and read in the usual fashion at room temperature (23°). The results are shown in Table I. It is evident that all the figures for a given concentration lie within the experimental error. Thus, changes in precipitation at temperatures between 20–30° are negligible.

The effect of temperature on the *final color* was studied on 1 ml. samples of the standard solutions described above, which were precipitated and

processed at 23°. Duplicate sets of the reagents used in color development and dilution (ammonium carbonate, hydrogen peroxide, distilled water) were carefully adjusted to 20° and to 30° and one group of precipitates developed at each temperature. The tubes were placed in constant temperature baths at these temperatures for 5 minutes more and then read colorimetrically. The results are shown in Table II. The variation of the meter readings is well within the limit of experimental error, indicat-

TABLE II

*Effect of Temperature on Developed Uranium Color Complex*

The figures represent actual meter readings.

Na concentration	20°	30°
<i>mg. per cent</i>		
250	34.3	34.7
300	29.2	29.0
350	25.0	24.9

TABLE III

*Reproducibility of Determinations in Standard Sodium (Chloride) Solutions*

	Na concentration, mg. per cent		
	250	300	350
Group A, Beckman spectrophotometer	249	301	352
	245	297	352
	246	301	346
	248	298	353
Group B, Leitz Rouy photometer		300	
		300	
		305	
		300	

ing that the effect of temperature variation between 20–30° on the final color is negligible.

### *Reproducibility*

The precision of the method was tested by quadruplicate determinations carried out on the 1:10 dilutions of the standards containing 250, 300, and 350 mg. per cent of sodium. The results are presented in Table III. It will be seen that, with one exception in Group A and one in Group B, the agreement of the figures is 1 per cent or better.

The data in Group A were obtained with a 1 cm. cell in the Beckman

spectrophotometer set at  $460\text{ m}\mu$ ; Group B was read in the Leitz Rouy photometer with a  $460\text{ m}\mu$  filter.

### Recovery

Recovery experiments were designed to approximate as closely as possible the conditions encountered in the handling of blood serum. For this

TABLE IV  
*Recovery of Sodium from Serum*

Sample	Meter reading	Na found <i>mg. per cent</i>	Na calculated <i>mg. per cent</i>	Error <i>per cent</i>	Recovery <i>per cent</i>
Dialyzed serum					
Run 1.....	50.9	136			
" 2.....	51.9	132			
" 3.....	51.5	134			
" 4.....	51.2	135			
Average .....		134			
Dialyzed serum + 150 mg. Na per 100 ml.			284		
Run 1.....	28.9	285		+0.35	
" 2.....	29.1	287		+1.06	
" 3.....	28.9	285		+0.35	
Average . . . . .		285.7		+0.57	100.6
Dialyzed serum + 200 mg. Na per 100 ml.			334		
Run 1.....	25.2	330		-1.19	
" 2.....	25.1	331		-0.9	
" 3.....	25.3	329		-1.5	
Average.....		330		-1.19	98.8

purpose, 400 ml. of fresh pooled serum were collected and dialyzed to remove a considerable proportion of the sodium. Analysis of a sample of the dialyzed specimen showed the residual sodium concentration to be 134 mg. per cent. To 100 ml. aliquots of this preparation, exactly 150 and 200 mg. of sodium (in the form of sodium chloride) were added, and the solutions were then analyzed for their sodium content at a concentration which now approximated the levels found in normal serum. The error of analysis and the recovery are shown in Table IV.



### Multiplier Effect

The multiplier effect is a well known device of the colorimetrist to increase the sensitivity of an instrument. It consists essentially of the substitution of a light-absorbing substance for the usual reagent blank. In this particular case, potassium dichromate solutions were found suitable for the purpose. Fig. 4 shows the effect on the calibration curve of the uranium complex when the aqueous ammonium carbonate reagent blank (Curve A) or dilute potassium dichromate solutions of 75 per cent (Curve B) or 50 per cent (Curve C) transmittance are used for the instrument blank. The curves show that a decrease in the transmittancy of the blank produces a decrease in the slope of the calibration curve (*i.e.*, an increase in sensi-

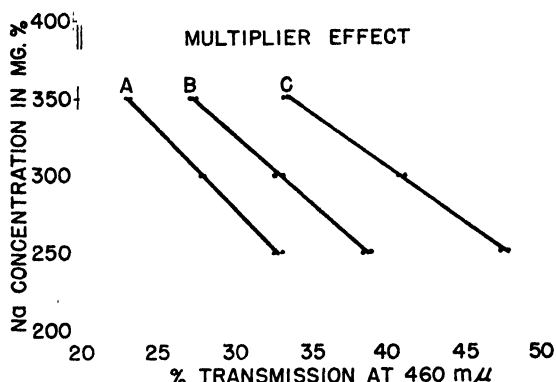


FIG. 4. Calibration curves obtained with instrument blanks of ammonium carbonate reagent (Curve A), and dilute potassium dichromate solutions of 75 per cent (Curve B) and 50 per cent (Curve C) transmittance.

tivity). In this particular case, the slope of the curve calibrated against ammonium carbonate was 1.0. With the 75 per cent transmittance blank the slope decreased to 0.86 and with the 50 per cent transmittance blank to 0.705. In practice, this means that a given range of concentrations is spread over a much larger portion of the meter scale, thus increasing the instrument sensitivity considerably.<sup>1</sup>

### SUMMARY

1. A rapid colorimetric method for the determination of sodium in biological fluids is presented. As outlined, it is specifically intended for the study of serum sodium.

2. Sodium is precipitated as the sodium-uranyl-zinc triple acetate. After washing, its color is intensified by application of the Rosenheim-Daehr reaction.

<sup>1</sup> The mathematical basis of this effect was presented recently by Hiskey (12).

3. The stability of the complex was studied as well as the effect of temperature upon it.

4. The accuracy of the method is within 1 per cent or better.

*Addendum*—It should be noted that when urine samples are prepared as outlined in our preliminary report (9), their sodium content may be determined equally well under the conditions given in this paper.

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# STUDIES ON THE MECHANISM OF NITROGEN STORAGE

## II. EFFECTS OF ANTERIOR PITUITARY GROWTH HORMONE PREPARATIONS ON KIDNEY GLUTAMINASE

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Glutamine has been suggested as an immediate source of nitrogen for the synthesis of essential nitrogenous components of protoplasm (1-3). Utilizing anterior pituitary growth hormone preparations for the production of nitrogen storage in dogs, we were unable to obtain any evidence that glutamine functions as an interim storage form of amino nitrogen (4). Changes produced in plasma glutamine and the excretion of urinary ammonia, however, emphasized the importance of the glutamine-glutaminase system in the transport and storage of ammonia (5) and suggested that kidney glutaminase might be directly or indirectly affected by anterior pituitary growth hormone.

Studies were therefore undertaken to determine whether or not growth hormone alters the activity of kidney glutaminase *in vitro*, or its concentration *in vivo*, in normal or hypophysectomized rats.

### EXPERIMENTAL

**Animals**—Female rats of the Sprague-Dawley strain, both normal and hypophysectomized, were used in all experiments. The hypophysectomized animals were supplied by the Hormone Assay Laboratories of Chicago and were 26 to 28 days old at the time of operation. The animals usually required from 10 to 14 days to reach a plateau in body weight after the operation. In some experiments, normal female rats 5 to 6 months old were used.

**Diets**—The rats were maintained on a diet consisting of 24 per cent casein, 55.5 per cent white flour, 10 per cent whole-milk powder, 1.5 per cent calcium carbonate, 1 per cent sodium chloride, 8 per cent corn oil supplemented with a vitamin mixture (6), and fresh lettuce.

**Growth Hormone Preparations**<sup>1</sup>—Preparations assayed by means of gain in weight of normal or hypophysectomized rats were used in all experiments. In some of the studies, preparations were used which had also been shown

<sup>1</sup> We are especially indebted to Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis and Company for supplying assayed preparations.

to produce both nitrogen storage and gain in weight in adult female dogs (4, 7).

**Methods**—Lyophilized growth hormone preparations were taken up in 0.8 per cent saline and prepared fresh at 2 day intervals. The solutions were stored frozen, and were thawed and warmed to 37° before administration. Injections were made once a day by the intraperitoneal route.

TABLE I

*Kidney Glutaminase of Normal and Hypophysectomized Rats and of Hypophysectomized Rats Treated with Anterior Pituitary Growth Hormone Preparations*

No. of rats	Female rats, 26-28 days old	Feeding method and treatment	Body weight			Glutaminase* per gm. kidney	†
			Initial	Terminal	Change		
			gm.	gm.	gm.	units	
6	Hypophysectomized	<i>Ad libitum</i> ; no treatment	72.0	73.5	1.5	76 ( $\pm 3.6$ )	
6	"	<i>Ad libitum</i> ; 10 mg. Preparation Rx-099816†	74.0	91.5	17.5	122 ( $\pm 2.4$ )	<0.01
5	Normal	Stomach tube;§ no treatment	99.0	92.5	-6.5	141 ( $\pm 26.2$ )	
5	"	<i>Ad libitum</i> ; no treatment	124.0	150.5	26.5	233 ( $\pm 12.2$ )	

\* The values shown are the average of two assays. Each assay was conducted on the pooled kidneys of two to three rats. The values in parentheses are the standard deviations of the mean.

† From Fisher's (14) table of *t*. Values of 0.01 or less are considered highly significant.

‡ Growth hormone, Preparation Rx099816, assayed at 1361 units per gm. in the normal adult female rat. The 10 mg. dose was administered in daily 1 mg. injections over a 10 day period. A 200 mg. dose of this preparation produced nitrogen storage of 0.7 gm. per kilo of body weight in an adult female dog (4).

§ Based on the average food consumption of the hypophysectomized rats treated with growth hormone.

In general, a 5 day control period followed by a 10 to 15 day period of treatment with the growth hormone preparation constituted an experiment. At the end of the experiment the rat was fasted for 24 hours and then sacrificed by a sharp blow on the head and decapitated. The kidneys were quickly removed, decapsulated, frozen, and stored in dry ice until assayed. Glutaminase assays were conducted according to the method of Archibald (8) by micro ammonia distillation and nesslerization (9).

### Results

In Table I are recorded the results of studies on kidney glutaminase of normal and hypophysectomized rats and of hypophysectomized rats treated

TABLE II

*Kidney Glutaminase of Hypophysectomized Rats Treated with Anterior Pituitary Growth Hormone Preparations*

Each group consisted of ten hypophysectomized female rats, 26 to 28 days old.

Feeding method and treatment	Body weight			Glutaminase* per gm. kidney
	Initial	Terminal	Change	
	gm.	gm.	gm.	units
<i>Ad libitum</i> ; no treatment	76.9	77.0	0.1	79 ( $\pm 10$ )
Pair-fed with untreated animals; 0.8 mg. Preparation Rx099816†	76.1	90.0	13.9	80 ( $\pm 4$ )

\* The values shown are the average of two glutaminase assays. Each assay was conducted on the pooled kidneys of five rats.

† Growth hormone, Preparation Rx099816, is the same preparation used and described in Table I.

TABLE III

*Kidney Glutaminase of Normal Rats Treated with Anterior Pituitary Growth Hormone Preparations*

Each group consisted of three normal female rats, 5 to 6 months old.

Feeding method and treatment	Body weight			Glutaminase* per gm. kidney
	Initial	Terminal	Change	
	gm.	gm.	gm.	units
Controls fed <i>ad libitum</i> ; no treatment	278.8	274.2	-4.6	95
Pair-fed with controls fed <i>ad libitum</i> ; 3.5 mg. Preparation Rx099916†	274.2	284.0	9.8	100
Pair-fed with controls fed <i>ad libitum</i> ; 7 mg. Preparation Rx099916	265.5	288.5	23.0	95

\* The values shown are for assays on pooled kidney preparations prepared from the number of rats shown in each group.

† Growth hormones, Preparation Rx099916, assayed at 3502 units per gm. in the normal adult female rat. In hypophysectomized rats this preparation assayed at an activity equivalent to about 91 per cent of that obtained at the 10  $\gamma$  level for a 10 day injection period with pure crystalline growth hormone described by Fishman, Wilhelmi, and Russell (15). The injections in this experiment were spread over a 15 day period.

with a growth hormone preparation. Treatment of hypophysectomized rats with growth hormone resulted in a marked increase in the concentration of kidney glutaminase, which approached the value obtained in nor-

mal rats fed the same amount of food by stomach tube. Normal rats of the same age group, fed *ad libitum*, gave a still higher assay of kidney glutaminase. Growth hormone-treated rats had an average daily food intake during the injection period of 5.9 gm., while the untreated hypophysectomized animals consumed 6 gm. per day.

TABLE IV

*Kidney Glutaminase of Normal Rats Treated with Anterior Pituitary Growth Hormone*  
Each group consisted of three normal female rats, 5 to 6 months old.

Feeding method and treatment	Body weight			Glutaminase* per gm. kidney	<i>p</i>
	Initial	Terminal	Change		
	gm.	gm.	gm.	units	
<i>Ad libitum</i> ; no treatment	258.3	256.3	-2.0	93 ( $\pm 10.0$ )	Between 0.2 and 0.1
Pair-fed with group fed <i>ad libitum</i> ; 20 mg. Preparation Rx099916†	259.8	297.5	37.7	83 ( $\pm 13.0$ )	

\* The values shown are the average of two assays. One assay was conducted on the pooled kidneys from two rats, while the other assay was conducted on the kidneys from one rat.

† Growth hormone, Preparation Rx099916, is the same as that described in Table III. The total dosage of 20 mg. was administered over a 15 day period.

TABLE V

*Effects In Vitro of Anterior Pituitary Growth Hormone Preparations on Kidney Glutaminase*

Growth hormone* added to assay tube	Amide nitrogen hydrolyzed in 15 min.
$\gamma$	$\gamma$
None	14.3
0.1	14.7
1.0	14.7
10.0	14.4
100.0	14.0
1000.0	15.0

\* Growth hormone, Preparation Rx099816, described in Table I, was used in this experiment.

In Table II are summarized the data obtained in similar experiments conducted at lower growth hormone dosage levels, with pair-fed hypophysectomized rats. It is quite apparent that, although the dosage levels employed were effective in producing body weight gains of from 17 to 19 per cent, no significant changes in kidney glutaminase were produced.

Table III shows the results of a study conducted on 5 to 6 month-old

normal female rats. Animals treated with doses of 3.5 and 7.0 mg. of growth hormone and pair-fed with controls receiving food *ad libitum* showed increases in weight but no significant change in glutaminase concentration.

In Table IV are shown data obtained at a higher level of growth hormone treatment. 5 to 6 month-old normal females, treated with a total dosage of 20 mg. of growth hormone and pair-fed with controls fed *ad libitum*, showed slightly diminished kidney glutaminase concentrations.

Table V summarizes the results of a typical study in which the effects of anterior pituitary growth hormone preparations were tested *in vitro* on kidney glutaminase preparations obtained from hypophysectomized rats. A series of assay tubes was prepared as in the usual assay procedure, and to each tube was added 1 ml. of an 0.8 per cent saline solution of the growth hormone preparation containing the desired concentration of the preparation to be tested. The tubes were incubated for 15 minutes and the amide nitrogen hydrolyzed was determined by the usual micro ammonia distillation procedure. Added concentrations of growth hormone ranging from 0.1  $\gamma$  to 1 mg. produced no significant effect.

#### DISCUSSION

From the results shown in Table I, it is apparent that hypophysectomy results in a decrease in the concentration of kidney glutaminase. Treatment of the hypophysectomized animals with growth hormone preparations at the total dosage level of 10 mg. produced a highly significant increase in kidney glutaminase, yet treatment with doses approximately one-tenth as great, as shown in Table II, failed to produce any change. Since treatment of the rats at the low dosage level produced a marked gain in weight, this response appears to be more sensitive than the increase in kidney glutaminase concentration.

Of considerable interest is the apparent increase in the utilization of food consumed by hypophysectomized rats treated with growth hormone. The food intake of both treated and untreated animals was nearly identical, yet the treated animals showed an increase in body weight of approximately 24 per cent above their control level. This finding is in agreement with results reported by Lee and Schaffer (10) and Nilson *et al.* (11) on normal pair-fed rats and on normal rats genetically equivalent with respect to the utilization of food.

No significant changes in kidney glutaminase concentrations were produced in 5 to 6 month-old normal female rats treated at three different dosage levels with a highly active growth hormone preparation. That this was not due to rapid excretion or inactivation of the hormone by normal animals is indicated by their gain in weight.



Failure to obtain significant effects from growth hormone preparations at physiological levels in experiments both *in vivo* and *in vitro* seems to indicate that anterior pituitary growth hormone has no direct effect on kidney glutaminase. The production of a significant increase in the concentration of kidney glutaminase in hypophysectomized animals at higher dosage levels, however, suggests an indirect effect. Explanation of such an effect might be found in an increased stimulation of renal glutaminase through ketosis (12), produced in rats treated with growth hormone (13).

#### SUMMARY

1. Effects of anterior pituitary growth hormone preparations on kidney glutaminase were studied in hypophysectomized animals, in normal animals, and in experiments *in vitro*.

2. Growth hormone preparations, at dosage levels well above those required to produce the physiological response of body weight gain, produce a significant increase in the concentration of kidney glutaminase in 26 to 28 day-old hypophysectomized rats. At dosage levels just sufficient to produce body weight gain, no change occurred in kidney glutaminase.

3. Anterior pituitary growth hormone had no effect on the kidney glutaminase of normal 5 to 6 month-old female rats which had reached a weight plateau.

4. Anterior pituitary growth hormone preparations produced no effect *in vitro* on the hydrolysis of amide nitrogen from glutamine by kidney glutaminase.

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# STUDIES ON THE MECHANISM OF NITROGEN STORAGE

## III. THE EFFECTS OF ANTERIOR PITUITARY GROWTH HORMONE PREPARATIONS ON THE PYRUVATE-ACTIVATED DEAMIDATION OF GLUTAMINE IN LIVER TISSUE

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High dehydropeptidase activity in neoplastic liver tissue (1) might be considered evidence for the active participation of dehydropeptides and their hydrolyzing enzymes in processes of growth. Since pyruvate-activated deamidation of glutamine has been interpreted as being due to intermediate dehydropeptide formation followed by enzymatic hydrolysis to glutamic acid, ammonia, and pyruvic acid (2), it seemed of interest to determine whether or not this process is altered in normal liver tissue when growth is arrested by hypophysectomy or induced by administration of growth hormone. Experiments of this type were also a logical extension of preceding ones concerning the glutamine-glutaminase system, in which deamidation is phosphate-activated (3).

### EXPERIMENTAL

*Tissues*—The rat liver tissues used in the deamidation studies were obtained from animals sacrificed during the course of the experiments reported in the preceding paper (3).

*Preparation of Tissue Extracts*—Aqueous extracts of fresh liver tissues were obtained by weighing the organ immediately after removal from the animal and homogenizing (4) with a volume of water equivalent to 10 times the organ weight. Homogenates were allowed to stand 1 hour at room temperature with occasional shaking and then centrifuged for 5 minutes at 1500 R.P.M. (5). The supernatant was used immediately in the deamidation study.

*Preparation of Reaction Tubes*—The reaction mixture used in determining the capacity of the liver extracts to deamidate glutamine in the presence of pyruvate was essentially the same as that described by Errera and Greenstein (6). Each tube contained 1 ml. of veronal-acetate buffer at pH 6.8, 1 ml. of aqueous glutamine substrate containing  $14\ \mu\text{M}$  of glutamine, 1 ml. of pyruvate solution neutralized to pH 7 and containing  $30\ \mu\text{M}$  per ml., and 0.5 ml. of rat liver extract. Extract and reagent blanks were prepared and run simultaneously. Tubes were incubated for 4 hours at  $38^\circ$ , and

then immediately frozen and stored in dry ice until the ammonia produced in the deamidation could be distilled. The micro ammonia distillation and nesslerization procedure of Archibald (7) was used. Results of the determination were expressed in terms of deamidation values defined as the number of micrograms of amide nitrogen (blank-corrected) split from glutamine under the described experimental conditions. The values tabulated, with the exception of those shown in Table II, are averages obtained from determinations conducted on liver extracts prepared from each rat in the group.

TABLE I

*Effects of Hypophysectomy and Growth Hormone Preparation on Pyruvate-Activated Deamidation of Glutamine in Liver Extracts*

No. of rats	Female rats, 26-28 days old	Feeding method and treatment	Body weight			Deamidation value,* amide N deamidated in 4 hrs.
			Initial	Terminal	Change	
			gm.	gm.	gm.	$\gamma$
5	Hypophysectomized	<i>Ad libitum</i> ; no treatment	72.8	73.4	0.6	23.7 ( $\pm 3.0$ )
5	Hypophysectomized	<i>Ad libitum</i> ; 10 mg. Preparation Rx099816†	74.0	92.2	18.2	22.9 ( $\pm 1.8$ )
4	Normal	Stomach tube, based on intake of treated rats	77.0	72.0	-5.0	27.3 ( $\pm 3.5$ )

\* The values in parentheses are standard deviations from the mean.

† Growth hormone, Preparation Rx099816, which assayed 1361 units per gm. by the normal adult female rat method. The rats received 1 mg. per day intraperitoneally for 10 days.

### Results

In Table I are recorded the results of a study on 26 to 28 day-old hypophysectomized rats treated with a total dose of 10 mg. of a growth hormone preparation previously shown to be potent with respect to the production of nitrogen storage and gain in body weight in the dog. Extracts prepared from the livers of the treated animals showed practically the same capacity to deaminate glutamine in the presence of pyruvate as extracts prepared from the untreated hypophysectomized group. Normal rats maintained at a food intake based on that of the treated group showed a slightly higher capacity to deaminate glutamine under the same conditions.

The results of a similar experiment conducted on four groups of rats in the same age group, but treated with much smaller doses of growth hormone, are summarized in Table II. Liver extracts prepared from hypophysec-

TABLE II

*Effects of Various Doses of Anterior Pituitary Growth Hormone Preparation on Pyruvate-Activated Deamidation of Glutamine in Liver Extracts*

Each group consisted of five females hypophysectomized at 26 to 28 days of age.

Feeding method and treatment	Body weight			Deamidation value,* amide N deamidated in 4 hrs.
	Initial	Terminal	Change	
	gm.	gm.	gm.	$\gamma$
<i>Ad libitum</i> ; saline controls	69.8	71.6	1.8	22.0
<i>Ad libitum</i> ; 10 $\gamma$ Preparation Rx099916 per day for 15 days†	67.0	77.9	10.9	31.2
<i>Ad libitum</i> ; 20 $\gamma$ Preparation Rx099916 per day for 15 days	69.0	85.1	16.1	25.2
<i>Ad libitum</i> ; 40 $\gamma$ Preparation Rx099916 per day for 15 days	68.7	89.3	20.6	20.0

\* Liver from five rats in each group pooled for determination of the deamidation value.

† Growth hormone, Preparation Rx099916, assayed at 3502 units per gm. by the normal adult female rat procedure. The total treatment was administered over a 15 day period. The assay of Preparation Rx099916, by the 26 to 28 day-old hypophysectomized rat procedure, showed an activity of approximately 91 per cent of that obtained at the 10  $\gamma$  per day injection level with pure crystalline growth hormone described by Fishman, Wilhelmi, and Russell (9).

TABLE III

*Effects of Anterior Pituitary Growth Hormone Preparation on Pyruvate-Activated Deamidation of Glutamine in Liver Extracts of Adult Rats*

Each group consisted of normal female rats, 5 to 6 months old.

No. of rats	Feeding method and treatment	Body weight			Deamidation value, amide N deamidated in 4 hrs.	$p^*$
		Initial	Terminal	Change		
		gm.	gm.	gm.	$\gamma$	
4	<i>Ad libitum</i> ; no treatment; controls	275.3	273.4	-1.9	29.6 ( $\pm 1.0$ )	
4	Pair-fed with controls; 3.5 mg. Preparation Rx099916†	270.1	281.0	10.9	32.9 ( $\pm 2.9$ )	0.6
3	Pair-fed with controls; 7.0 mg. Preparation Rx099916	261.8	284.8	23.0	32.3 ( $\pm 9.3$ )	

\* From Fisher's table of  $t$  values. A value of 0.01 or less is highly significant.

† Growth hormone, Preparation Rx099916, described in Table II. Injections were spread over a 15 day period.

tomized rats treated with 10  $\gamma$  of growth hormone per day for 15 days showed a greater capacity to deamidate glutamine than liver extracts from similar rats treated with 20 and 40  $\gamma$  per day.

Tables III and IV show the results of studies on 5 to 6 month-old normal female rats, treated at three different dosage levels with the growth hormone preparation described in Table II. The capacity of liver extracts prepared from animals treated at the 3.5 and 7.0 mg. levels to deamidate glutamine in the presence of pyruvate does not differ significantly from liver extracts of normal controls. At the 20 mg. level of treatment the difference in the deamidating capacity is greater but still statistically insignificant.

TABLE IV

*Effects of Large Doses of Anterior Pituitary Growth Hormone Preparation on Pyruvate-Activated Deamidation of Glutamine in Liver Extracts*

Each group consisted of four normal female rats, 5 to 6 months old.

Feeding method and treatment	Body weight			Deamidation value, amide N deamidated in 4 hrs.	$\bar{x}$
	Initial	Terminal	Change		
	gm.	gm.	gm.	$\gamma$	
<i>Ad libitum</i> ; no treatment; controls	258.2	256.3	-1.9	32.4 ( $\pm 2.5$ )	0.4
Pair-fed with controls; 20 mg. Preparation Rx099916*	259.8	297.5	37.7	38.0 ( $\pm 2.3$ )	

\* Growth hormone, Preparation Rx099916, described in Table II. Injections were spread over a 15 day period.

#### DISCUSSION

In considering the results of our experiments on both normal and hypophysectomized rats, we find that pyruvate-activated deamidation of glutamine in liver extracts is in general unaffected by hypophysectomy or by anterior pituitary growth hormone preparation, even though decided changes in weight were produced. The results of the study tabulated in Table II might suggest an effect at very low levels of hormone treatment. Liver extracts prepared from rats treated with 10  $\gamma$  per day gave a deamidation value of 31.2, while those from livers of control animals gave a value of 22.

In addition to the proposed dehydropeptide-dehydropeptidase mechanism for pyruvate-activated deamidation of glutamine (2), it recently has been suggested that pyruvate might function as a coenzyme in a reversible enzyme-pyruvate complex (8). Regardless of the mechanism, our negative results are of interest in that pyruvate-activated deamidation of glutamine in rat liver tissue appears to be unaffected by growth hormone at doses above 10  $\gamma$  per day.

#### SUMMARY

1. Effects of anterior pituitary growth hormone preparations on the pyruvate-activated deamidation of glutamine were studied in liver tissue extracts prepared from both normal and hypophysectomized rats.

2. Treatment of hypophysectomized rats at high dosage levels had no effect on the capacity of the liver tissue extracts to deamidate glutamine in the presence of pyruvate. Results of studies at very low levels of growth hormone treatment suggest an effect but are not conclusive.

3. The capacity of the normal rat liver to deamidate glutamine in the presence of pyruvate appears to be unaffected by anterior pituitary growth hormone treatment. Three different levels of growth hormone treatment were studied.

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# THE SEPARATION OF DEHYDROPEPTIDASE AND ANALOGOUS L- AND D-PEPTIDASES\*

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The three peptides, glycyldehydroalanine (GDA), glycyl-L-alanine (L-GA), and glycyl-D-alanine (D-GA), are rapidly hydrolyzed by kidney and other tissues (2-6). Thus far no known function has been demonstrated for the so called D-peptidase and dehydropeptidase systems. The problem arises as to whether the enzymes attacking these three analogous substrates are separate in identity. Maschmann has shown that in guinea pig kidney extracts manganese and cysteine, when added together, markedly accelerate the cleavage of the more slowly hydrolyzed D isomer of glycyl-DL-alanine, whereas the more rapidly hydrolyzed L isomer was slightly inhibited (6). On the basis of these experiments he has concluded that the two isomers of glycyl-DL-alanine are hydrolyzed by different enzymes.

Using glycyldehydroalanine and optically pure glycyl-L-alanine and glycyl-D-alanine (7), we have utilized a number of different experimental techniques to investigate this problem more fully. The methods used have included (a) a comparison of the activity ratios of the three substrates in extracts of rat tissue, (b) differential centrifugation of tissue homogenates, (c) alcohol, low temperature fractionation, (d) the relation of activity to pH, (e) enzyme saturation studies, and (f) activation and inhibition experiments. Evidence from all of these approaches is consistent with the conclusion that the hydrolysis of each of the three substrates is catalyzed by a separate enzyme.

## EXPERIMENTAL

### *Preparation of Substrates*

Glycyldehydroalanine was obtained by amination of chloroacetyldehydroalanine (8). Chloroacetyldehydroalanine was synthesized from chloroacetonitrile and pyruvic acid by the method of Price and Greenstein (9).

Glycyl-L-alanine and glycyl-D-alanine were prepared by the enzymatic resolution of chloroacetyl-DL-alanine by the procedure of Fodor, Price, and Greenstein (7).

\* A preliminary report of this work has appeared (1).



*Enzymatic Determinations*

Dehydropeptidase activity was determined by the measurement of the disappearance of the characteristic ultraviolet absorption spectrum of glycyldihydroalanine (10),<sup>1</sup> or by determination of the ammonia evolved during the digestion. Hydrolysis of glycyllalanine was measured by the estimation of the amino acids released by using the Van Slyke gasometric ninhydrin procedure (11).

The digests generally consisted of 1 cc. of tissue extract<sup>2</sup> or enzyme solution in suitable dilution, 2 cc. of borate or veronal buffer at the desired pH, and 1 cc. of 0.05 M substrate. Correction was made for the enzyme blanks. There was no significant non-enzymatic hydrolysis of the substrates under the conditions of the determinations.

The rate of enzymatic activity toward the three substrates is expressed in terms of micromoles of peptide hydrolyzed per hour per mg. of protein nitrogen, and is designated as Ac(L-GA), Ac(D-GA), and Ac(GDA). The rates were approximated from the initial portion of the respective time curves. An alternative abbreviation for the activity of the respective enzymes is L-GAase, D-GAase, and GDAase.

*Tissue Distribution Studies*

A number of tissues were ground with sand and extracted with 5 volumes of 0.15 M borate buffer at pH 8.0. After light centrifugation the extracts were diluted as necessary and assayed immediately. The activities are given in Table I.

All three substrates were hydrolyzed most rapidly by kidney. L-GA had the greatest susceptibility in all the tissues except defibrinated blood. Although both L-GA and GDA were hydrolyzed by all the tissues studied, there was no measurable hydrolysis of D-GA by hog spleen or by rat brain, muscle, and defibrinated blood. The ratios of the rates of hydrolysis of any two of the three substrates are not constant from one rat tissue to another. The ratio of Ac(L-GA):Ac(D-GA) varies from 8:1 in pancreas to 164:0 in brain, while Ac(L-GA):Ac(GDA) varies from 3:1 in pancreas to 12:1 in muscle. The ratio of Ac(GDA):Ac(D-GA) is 2:1 in kidney and 18:0 in brain. These variations in ratios from one tissue to another suggest the possibility that these substrates may be hydrolyzed

<sup>1</sup> The measurements were made at 2400 Å on aliquots of digests deproteinized with 3 per cent perchloric acid and read against controls in which the substrate was omitted.

<sup>2</sup> Hog and beef tissues were obtained fresh from a slaughter-house, frozen immediately, and stored at -15° for not more than 1 week before use. Some variation was found from one batch of tissues to another, depending presumably upon the age and condition of the animals.

by different enzymes. The absence of D-GAase in brain, muscle, and defibrinated blood is especially significant, for each of these tissues has a definite L-GAase and GDAase.

### *Differential Centrifugation*

Rat tissues were ground with 6 volumes of ice-cold water in a Potter-Elvehjem homogenizer and centrifuged at  $26,000 \times g$  for 2 hours at  $0^\circ$ . The sediments and supernatants were diluted to the original volume and assayed for the respective peptidase activities. In every tissue

TABLE I  
*Hydrolysis of Analogous Peptides by Crude Tissue Extracts*

Tissue	Substrate hydrolyzed per hr. per mg. N*		
	Glycyl-L-alanine	Glycyl-D-alanine	Glycyl-D-hydroalanine
	$\mu M$	$\mu M$	$\mu M$
Rat kidney.....	1240	136	256
" liver.....	341	2.9	29
" lung.....	493	59	116
" spleen.....	313	0.7	24
" testicle.....	162	17	50
" brain.....	164	0	18
" pancreas.....	116	15	36
" muscle.....	47	0-	3.8
" blood.....	4.6	0	5.1
Hog kidney.....	4350	310	508
" spleen.....	380	0	39
" pancreas.....	103	38	27
Mushrooms.....	204	0.5	21

\* At  $38^\circ$ ; the digests consisted of 1 cc. of tissue extract, 2 cc. of 0.15 M borate buffer at pH 8.0, and 1 cc. of 0.05 M substrate.

(Table II) the L-GAase was predominantly in the supernatant and only 10 to 20 per cent was found in the unwashed sediment. With D-GAase the reverse situation holds, for here in all the tissues studied the greater percentage of activity was found in the sediment.

It is apparent from these data that the L-GAase is largely present in tissue homogenates as a soluble enzyme, although even on repeated washing all of the activity cannot be removed from the sediment. The fact that most of the D-GAase can readily be separated from the soluble L-GAase by centrifugation makes it quite certain that these activities represent two separate enzymes. The particulate nature of D-GAase is of interest and further study of this finding is in progress. Preliminary

experiments indicate that in rat kidney and hog pancreas homogenates the D-GAase is concentrated primarily in the small particles, sedimentable between  $9000 \times g$  and  $26,000 \times g$ .<sup>3</sup>

In all the tissues the greater part of GDAase was in the supernatant with the exception of kidney, where 85 to 90 per cent of the activity was

TABLE II

*Separation of Activities toward Three Analogous Peptides by Centrifugation at  $26,000 \times g$ \**

Rat tissue	Substrate	Per cent of original activity		
		Supernatant	Pellet	Sum
Kidney . . . . .	L-GA	51	21	72
	D-GA	7	80	87
	GDA	25	88	113
Liver . . . . .	L-GA	61	8	69
	D-GA	17	79	96
	GDA	82	21	103
Lung . . . . .	L-GA	96	13	109
	D-GA	25	89	114
	GDA	80	32	112
Spleen . . . . .	L-GA	88	12	100
	D-GA	24	78	102
	GDA	74	24	98
Pancreas . . . . .	L-GA	111	13	124
	D-GA	46	76	122
	GDA	81	16	97
Testis . . . . .	L-GA	100	12	112
	D-GA	15	106	121
	GDA	98	5	103
Brain . . . . .	L-GA	85	14	99
	D-GA	0	0	0
	GDA	79	16	95

\* The digests consisted of 1 cc. of enzyme solution, 1 cc. of 0.15 M borate buffer at pH 8.0, 1 cc. of water, or 0.05 M substrate.

consistently found in the sediment. Attempts to separate GDAase from D-GAase by differential centrifugation of rat kidney homogenates

<sup>3</sup> Rat kidney was homogenized with 5 volumes of ice-cold water and the homogenate centrifuged at  $9000 \times g$  for 20 minutes. The sediment (large particles) had an Ac(D-GA) of 1.5 and an Ac(GDA) of 12. The supernatant,  $S_1$ , was centrifuged at  $26,000 \times g$  for 2 hours. The sediment (small particles) had an Ac(D-GA) of 140 and an Ac(GDA) of 104. The final supernatant,  $S_2$ , had an Ac(D-GA) of 8.5 and an Ac(GDA) of 21. Thus when the activity of the various fractions is related to their nitrogen content, it is apparent that the most active fraction is that of the small particles with respect to both D-GA and GDA.

have been unsuccessful. It is apparent that the GDAase is also associated with the small particles,<sup>3</sup> and that, although on centrifugation at varying speeds from  $8000 \times g$  to  $25,000 \times g$  moderate changes in ratio between the two activities are obtained, they are not readily reproducible. It seems probable that, while the small particles are not homogeneous in their enzyme content, separation of the two activities will be very difficult by available techniques.

#### *Alcohol, Low Temperature Fractionation*

Hog spleen has no measurable capacity to hydrolyze D-GA. This tissue was subjected to alcohol, low temperature fractionation to attempt separation of the GDAase and L-GAase. This has been accomplished, although with some difficulty because of the lability of the two enzymes and because of the similarity in solubility of the two proteins under a variety of conditions. In homogenates of hog spleen the ratio of  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  was about 8:1. By the following procedure we have obtained a preparation which is low in L-GAase as compared to GDAase. During the course of the fractionation the ratio of  $\text{Ac(L-GA)}:-\text{Ac(GDA)}$  was changed 70-fold.

6700 gm. of thawed, defatted, fresh frozen hog spleen were ground in a Waring blender with 2 volumes of ice-cold water. The mixture stood in the cold for 2 hours and was filtered through gauze. Sodium chloride was added to 0.1 M concentration. The activities of the various fractions are summarized in Table III. Total activities, designated as  $\text{TAc(L-GA)}$  and  $\text{TAc(GDA)}$ , are expressed in units calculated so that 1 unit of peptidase activity is the amount of enzyme necessary to hydrolyze  $1 \mu\text{M}$  of substrate per hour. The original extract had a volume of 18,000 cc., a nitrogen content of 5.6 mg. per cc., a  $\text{TAc(L-GA)}$  of 27,000,000 units, and a  $\text{TAc(GDA)}$  of 3,460,000 units. This extract was centrifuged for 20 minutes at  $600 \times g$  and then put through a Sharples-refrigerated centrifuge twice at  $60,000 \times g$  (the radius measured to the outer wall of the centrifuge bowl). The sediment,  $P_1$ , was discarded. The clear supernatant,  $S_1$ , had a volume of 12,800 cc. and nitrogen of 3.2 mg. per cc. The  $\text{TAc(L-GA)}$  was 17,000,000 units and the  $\text{TAc(GDA)}$  was 1,960,000 units. Cold 60 per cent alcohol was added to  $S_1$  to a final concentration of 8 per cent. 0.2 M acetic acid was added to lower the pH to 6.3. The sodium chloride concentration was kept at 0.1 M through  $P_2$  (Table III), after which no sodium chloride was added. After 12 hours at  $-4^\circ$  the precipitate,  $P_2$ , was collected at the Sharples centrifuge, care being taken to keep the temperature just above the freezing point of the solution.  $P_2$  had a  $\text{TAc(L-GA)}$  of 1,700,000 and a  $\text{TAc(GDA)}$  of 120,000 units. The  $\text{TAc(L-GA)}$  of  $S_2$  was 15,000,000 units,

the TAc(GDA) 1,800,000 units.  $S_2$  was taken to 12 per cent alcohol, pH 5.3,  $-6^\circ$  for 12 hours. The precipitate,  $P_3$ , was discarded and the supernatant,  $S_3$ , was adjusted to 30 per cent alcohol at pH 5.0, and  $-15^\circ$  for 12 hours to precipitate the active protein, so that it could be resuspended in a smaller volume. After being taken up in 0.1 M NaCl, the ex-

TABLE III  
*Summary of Hog Spleen Fractionation*

Fraction	Conditions	Substrate hydrolyzed per hr. per mg. enzyme N*		Ratio, Ac(L-GA): Ac(GDA)
		Glycyl-L- alanine	Glycyl- dehydro- alanine	
Original.....	0.1 M NaCl	$\mu M$	$\mu M$	
$S_1$ .....	Centrifugation	270	34	7.9
$P_1$ (discarded).....		412	47	8.7
$S_2$ .....	8% alcohol, pH 6.3, $-4^\circ$	412	50	8.2
$P_2$ (discarded).....		291	22	14.3
$S_3$ .....	12% " " 5.3, $-6^\circ$	103	25	4.1
$P_3$ (discarded).....		586	47	12.5
$S_4$ ".....	30% " " 5.3, $-15^\circ$			
$P_4$ .....		149	43	3.4
$S_5$ .....	12% " " 5.3, $-6^\circ$	15	15	1.0
$P_5$ (discarded) ..		29	5.1	5.7
$S_6$ ".....	30% " " 5.6, $-15^\circ$			
$P_6$ .....		36	18	2.0
$S_7$ .....	12% " " 5.9, $-6^\circ$	25	47	0.52
$P_7$ (discarded) ..		36	12	3.00
$S_8$ ".....	30% " " 5.0, $-15^\circ$			
$P_8$ .....		27	38	0.70
$S_9$ .....	12% " " 5.9, $-6^\circ$	32	112	0.28
$P_9$ (discarded) ..		24	15	1.6
$S_{10}$ ".....	30% " " 5.0, $-15^\circ$			
$P_{10}$ .....		24	104	0.24
$S_{11}$ .....	12% " " 5.5, $-6^\circ$	15	73	0.20
$P_{11}$ (discarded).....		28	28	1.0

\* The conditions of the assay are the same as in Table II.

tract,  $P_4$ , had a protein nitrogen concentration of 3.0 mg. per cc. and was taken again to 12 per cent alcohol, pH 5.3,  $-6^\circ$ . The supernatant  $S_4$  had 0.62 mg. of N per cc. and was discarded. These alternate 12 per cent and 30 per cent alcohol steps were repeated three more times. The final clear, slightly pink supernatant,  $S_{11}$ , had a volume of 45 cc. and a nitrogen content of 1.1 mg. per cc., with a TAc(L-GA) of 730 and a TAc(GDA) of 3600 units.

$P_2$  had a ratio of  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  of 14.2:1.  $S_{11}$  had a ratio of 0.203:1, so that during the course of the fractionation the ratio was markedly reversed and a 70-fold change in ratio was achieved. Fraction  $P_2$  was used for many of the studies that follow.

The change in ratio by means of the fractionation procedure described indicates the presence of two different enzymatic entities. A high concentration of the enzyme hydrolyzing glycyldehydroalanine could not be obtained. Fraction  $P_2$  had high GDAase but was discarded because the ratio  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  was even higher than the original homogenate. In obtaining the marked changes in activity ratios a less active fraction on the basis of activity per mg. of protein N resulted.

That the separation is not simply due to denaturation of the L-GDAase may be seen from the continuous progressive change in ratio throughout the fractionation procedure. The activity values of fractions  $P_2$ ,  $S_2$ , and  $P_3$  bear directly on this point. Fraction  $P_2$  had a  $\text{TAc(L-GA)}$  of 13,000 and a  $\text{TAc(GDA)}$  of 18,600 with a ratio of  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  of 0.70:1. This was taken to 12 per cent alcohol, pH 5.9,  $-6^\circ$ , and after 12 hours centrifuged. The supernatant,  $S_2$ , had a  $\text{TAc(L-GA)}$  of 3750 and a  $\text{TAc(GDA)}$  of 13,000 with a ratio of 0.28:1, whereas the precipitate,  $P_3$ , had a  $\text{TAc(L-GA)}$  of 8500 and a  $\text{TAc(GDA)}$  of 5300 with a ratio of 1.6:1. If the activities of  $S_2$  and  $P_3$  are added together, we find a recovery of 12,250 units of  $\text{TAc(L-GA)}$  out of 13,000 in  $P_2$ , or a 94 per cent recovery of the  $\text{TAc(L-GA)}$ . 18,300 out of 18,600 units of GDAase, or 98 per cent, were recovered. In this and in some of the other steps there was only slight loss of activity, while in still others the loss in activity was marked. Regardless of this, in each step the ratio  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  was higher in the pellet of the 12 per cent alcohol step, and consistently lower in the supernatant. Since hog spleen has no measurable D-GAase and fraction  $S_{11}$  has an  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  ratio of 0.2,  $S_{11}$  is at least 5 times higher in its rate of hydrolysis of GDA than either of the two saturated peptides.

### *pH-Activity Studies*

With rat kidney homogenates the optimal pH is 8.3 for the hydrolysis of L-GA, 8.2 for GDA, and 8.8 for D-GA.

A study of the relation between pH and the rate of hydrolysis of L-GA and GDA in hog spleen fraction  $P_2$  revealed striking differences. The optimal pH for the hydrolysis of L-GA is 8.2, whereas that for GDA is 7.7. In Fig. 1 it may be noted that in this fraction from pH 6 to pH 7.2 the ratio of  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  is 1:1. At pH 8.0 this ratio rises to 1.7:1 and at pH 8.5 it is 6.2:1. At pH 9.0 the ratio of activities is 14:1 and the hydrolysis of GDA is minimal, whereas that of L-GA is still at 70

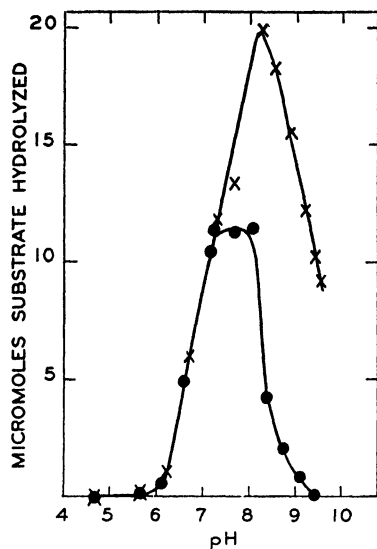


FIG. 1. Hydrolysis at different pH values of glycyl-L-alanine (X) and glycyldehydroalanine (●) by hog spleen fraction  $P_4$ . The digests consisted of 1 cc. of  $P_4$  diluted 1:5 with water, 2 cc. of buffer, and 1 cc. of either water or 0.05 M substrate.  $P_4$  had a protein nitrogen of 7.7 mg. per cc. From pH 4.6 to 6.2 a 0.0237 M veronal buffer was used. Above pH 7.0, Sørensen's borate buffer was used. Incubation period, 20 minutes at 38°.

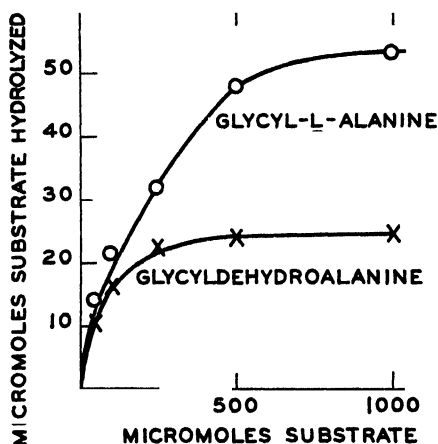


FIG. 2. Substrate saturation curves of glycyl-L-alanine (O) and glycyldehydroalanine (X) in hog spleen fraction  $P_4$ . The digests consisted of 1 cc. of fraction  $P_4$  diluted 1:6 with water, 2 cc. of Sørensen's borate buffer, and 1 cc. of solution containing 0.05, 0.1, 0.25, 0.5, and 1.0 M substrate for the successive points. Substrate and borate were added together and adjusted to 7.4. The enzyme was separately adjusted to 7.4. Incubation period, 20 minutes at 38°.

per cent of the maximal rate. This is again strongly suggestive that we are dealing with two separate enzymatic activities.

### *Effect of Substrate Concentration on Rate of Hydrolysis*

The effect of substrate concentration on the rate of hydrolysis of L-GA and GDA by fraction P<sub>6</sub> of hog spleen is shown in Fig. 2. With the same amount of P<sub>6</sub> in the digestion mixture considerably more L-GA than

TABLE IV  
*Activation-Inhibition Studies with Rat Kidney*

Compound	Substrate hydrolyzed per hr. per mg. N*		
	Glycyl-L-alanine	Glycyl-D-alanine	Glycyldehydroalanine
	$\mu M$	$\mu M$	$\mu M$
None . . . . .	916	42	190
Zn <sup>++</sup> acetate† . . . . .	41	66	371
Co <sup>++</sup> " †† . . . . .	418	100	295
Mn <sup>++</sup> Cl <sub>2</sub> ‡ . . . . .	685	49	212
Mg <sup>++</sup> Cl <sub>2</sub> ‡ . . . . .	896	39	189
Cysteine HCl§ . . . . .	47	9	73
" § + MnCl <sub>2</sub> ‡ . . . . .	690	45	236
KCN   . . . . .	333	9	54
Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub>    . . . . .	49	8	50

\* The conditions of the assay are the same as in Table II. -Activator or inhibitor was added to the buffer and carefully adjusted to pH 8.0.

† Final concentration, 0.001 M.

‡ It was noted that on adding cobaltous ion to glycyldehydroalanine the solution became yellow-brown and then deep pink in color. This is probably due to complex formation between cobaltous ion and glycyldehydroalanine. Burk *et al.* have demonstrated similar color changes in the formation of complexes between cobaltous ion and peptides of histidine (12). Further work on this observation is in progress.

§ Final concentration, 0.003 M.

|| Final concentration, 0.01 M.

GDA is required for saturation. The Michaelis-Menten constant for GDAase is  $16.5 \times 10^{-3}$  mole per liter and that of L-GAase,  $47.0 \times 10^{-3}$  mole per liter.

### *Activators and Inhibitors*

The effect of several metallic salts, cysteine, cyanide, and pyrophosphate in crude rat kidney extracts was examined. The compound to be studied was added to the 0.15 M borate buffer and the pH carefully adjusted to 8.0. Determinations of the pH of the digestion mixture were checked before and after incubation. In Table IV it will be seen that



there was marked inhibition of hydrolysis of all three substrates by cysteine, cyanide, and pyrophosphate. Manganese ion strongly counteracts the cysteine inhibition with each of the substrates. In contrast to the finding of Maschmann with guinea pig kidney extracts, we observed no marked activation of D-GAase by  $Mn^{++}$  and cysteine in rat kidney.  $Zn^{++}$  causes marked inhibition of the soluble L-peptidase but activates both the D-GAase and the GDAase. Yudkin and Fruton (13) have reported a similar  $Zn^{++}$  activation of the hydrolysis of glycyldehydrophenylalanine in swine kidney.  $Co^{++}$  causes moderate inhibition of the L-peptidase and activates D-GAase and GDAase.  $Mn^{++}$  inhibits

TABLE V  
*Effect of  $Zn^{++}$  Ion on Enzymatic Hydrolysis of Glycyl-L-alanine and Glycyldehydroalanine*

Tissue	Substrate hydrolyzed per hr. per mg. extract N*			
	Glycyl-L-alanine		Glycyldehydroalanine	
	Without $Zn^{++}$	With $Zn^{++}\dagger$	Without $Zn^{++}$	With $Zn^{++}\dagger$
	$\mu M$	$\mu M$	$\mu M$	$\mu M$
Rat kidney	1010	70	190	484
" spleen . . . . .	286	0	33	23
Hog kidney . . . . .	2535	462	387	687
" spleen . . . . .	1246	0	95	4
Beef kidney . . . . .	2380	70	404	1640
" spleen	324	11	25	4

\* The conditions of the assay are the same as in Table IV.

† Final concentration, 0.001 M.

slightly the L-peptidase, but otherwise  $Mn^{++}$  and  $Mg^{++}$  have no significant effect.

Care should be exercised in interpreting the results of activation studies on enzymes whose activities are primarily located in the cell particulate matter. In the case of  $Zn^{++}$  the activity of the soluble L-GAase was strongly inhibited but the D-GAase and GDAase were accelerated. Both of the latter two activities are in the small particles. In view of this difference in the physical state of the enzymes it is difficult, at the present time, to interpret and to utilize this difference in activation as being more than suggestive evidence for the separate identity of L-GAase from the D-GAase and the GDAase. The data in Table V emphasize this point. GDAase, like L-GAase, is a soluble protein in rat, hog, and beef spleen. The addition of  $Zn^{++}$  causes slight inhibition of GDAase in rat spleen and marked inhibition in both hog and beef spleen. This is in contrast with the finding of marked activation by  $Zn^{++}$  of the insoluble

ble GDAase in rat, hog, and beef kidney (Table V). The mechanism of this  $\text{Zn}^{++}$  activation of the GDAase in kidney extracts is still unknown (*cf.* (13)).

In the study of hog spleen fraction  $P_6$  it was found that 0.003 M cysteine caused marked inhibition of L-GAase, whereas the GDAase was only minimally affected. The relation of this inhibition to pH is shown in Table VI. The ratio of  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  rises as the pH is raised from 6.9 to 8.2, both with and without cysteine. This is consistent with the pH-activity study already described. The cysteine inhibition of L-GAase at pH 6.9, 7.4, and 8.2 is 60, 57, and 54 per cent respectively. Under identical conditions the inhibition of GDAase is only 19, 35, and 0

TABLE VI

*Effect of Cysteine on Hydrolysis of Glycyl-L-alanine and Glycyldehydroalanine under Identical Conditions by Hog Spleen Preparation\**

Substrate	pH	Substrate hydrolyzed		Ratio, $\text{Ac(L-GA)}:\text{Ac(GDA)}$	
		No cysteine	With cysteine†	No cysteine	With cysteine†
		$\mu\text{M}$	$\mu\text{M}$		
Glycyl-L-alanine.....	6.9	18.7	7.4	1.2	0.6
Glycyldehydroalanine. . . . .	6.9	14.9	12.1		
Glycyl-L-alanine.....	7.4	29.1	12.5	1.3	0.8
Glycyldehydroalanine . . . . .	7.4	23.3	15.0		
Glycyl-L-alanine.....	8.2	43.8	20.3	4.2	1.8
Glycyldehydroalanine . . . . .	8.2	10.4	11.0		

\* Digests consisted of 1 cc. of fraction  $P_6$ , diluted 1:6 with  $\text{H}_2\text{O}$ , 2 cc. of borate buffer, and 1 cc. of 0.25 M substrate. Incubation period 20 minutes at 38°.

† Final concentration, 0.003 M.

per cent respectively. These data were rechecked with quadruplicate determinations and a carefully controlled pH of 7.4 with 0.25 M substrate. The results obtained were 53 per cent inhibition of the L-GAase and 15 per cent inhibition of the GDAase. The difference in cysteine inhibition is also present when the enzyme is fully saturated by substrate. When 1 cc. of 1.0 M L-GA was present in the 4 cc. digestion mixture at pH 7.4, the hydrolysis of L-GA was 41.3 and 42.1  $\mu\text{M}$  in duplicate determinations, whereas with cysteine it was 13.1 and 12.8  $\mu\text{M}$ . With 1.0 M GDA under identical conditions the values were 22.8 and 21.8  $\mu\text{M}$  without cysteine, and 20.2 and 20.3  $\mu\text{M}$  with cysteine. In the absence of cysteine the ratio  $\text{Ac(L-GA)}:\text{Ac(GDA)}$  was 1.9, whereas in the presence of a final concentration of 0.003 M cysteine the ratio was 0.6. The addition of cysteine by its marked inhibition of L-GAase caused a com-

plete reversal of the activity ratio. Thus it seems highly probable that the hydrolysis of L-GA and GDA is catalyzed by separate enzymes.

We are indebted to Mrs. Mary Hoyer and Miss Florence Leuthardt for technical assistance.

#### SUMMARY

1. Different experimental techniques have been utilized to demonstrate that glycyl-L-alanine, glycyl-D-alanine, and glycyldehydroalanine are hydrolyzed by different enzymes.

2. The ratio of the rates of hydrolysis of any two of the three substrates by extracts of rat tissue is inconstant from one tissue to another. There is no measurable hydrolysis of glycyl-D-alanine by rat brain and muscle, whereas both glycyl-L-alanine and glycyldehydroalanine are hydrolyzed by these tissues.

3. Differential centrifugation studies revealed that the enzyme hydrolyzing glycyl-L-alanine is primarily a soluble protein, while the enzyme hydrolyzing glycyl-D-alanine is in the particulate matter. The enzyme attacking glycyldehydroalanine is soluble except in kidney, where it is in the particulate matter.

4. By alcohol, low temperature fractionation of a hog spleen homogenate the ratio of the rate of hydrolysis of glycyl-L-alanine to glycyldehydroalanine was changed from 14:1 to 0.2:1 during the course of the fractionation. Since hog spleen does not attack glycyl-D-alanine, the final preparation,  $S_{11}$ , was higher in its activity toward glycyldehydroalanine than either of the analogous saturated peptides.

5. In rat kidney the pH of optimal activity toward glycyl-L-alanine is at 8.3, toward glycyl-D-alanine at 8.8, and toward glycyldehydroalanine at 8.2. In a hog spleen fraction,  $P_6$ , the pH of optimal activity toward glycyl-L-alanine is at 8.2, while that toward glycyldehydroalanine is at 7.7. The dissimilarity between the pH activity curves is discussed.

6. Enzyme saturation studies were carried out on fraction  $P_6$  of hog spleen. The Michaelis-Menten constant of glycyl-L-alanine was  $47 \times 10^{-3}$  mole per liter and that of glycyldehydroalanine was  $16.5 \times 10^{-3}$  mole per liter.

7. In activation and inhibition experiments on rat kidney extracts it was found that 0.003 M cysteine, 0.01 M cyanide, and 0.01 M pyrophosphate markedly inhibit the enzymatic hydrolysis of all three substrates.  $Mn^{++}$  largely counteracted the cysteine inhibition but in the absence of cysteine had little effect.

$Zn^{++}$  and  $Co^{++}$  inhibit the hydrolysis of glycyl-L-alanine by rat kidney extracts, whereas the hydrolysis of glycyl-D-alanine and glycyldehydroalanine is accelerated.

8. In rat, hog, and beef kidney extracts, where the enzymatic activity toward glycyldehydroalanine is in the particulate matter, the addition of  $\text{Zn}^{++}$  markedly accelerates the hydrolysis of glycyldehydroalanine. In contrast to this in extracts of rat, hog, and beef spleen the enzyme attacking glycyldehydroalanine is a soluble protein. There is slight inhibition of the hydrolysis of glycyldehydroalanine by  $\text{Zn}^{++}$  in extracts of rat spleen and very marked inhibition by  $\text{Zn}^{++}$  in extracts of hog and beef spleen.

Caution is recommended in the interpretation of the results of activation studies on enzymes located in the particulate fractions.

9. Cysteine causes marked inhibition of the hydrolysis of glycyl-L-alanine in hog spleen, fraction P<sub>6</sub>, under conditions in which the cleavage of glycyldehydroalanine is only slightly inhibited.

10. These findings are consistent with the conclusion that glycyl-L-alanine, glycyl-D-alanine, and glycyldehydroalanine are each hydrolyzed by a separate enzyme.

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# SEPARATION OF ENZYMATIC ACTIVITIES TOWARD CHLOROACETYLALANINE, CHLOROACETYL- ALANYLGLYCINE, AND GLYCYLALANINE

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*N*-Acylated amino acids and *N*-acylated dipeptides are readily hydrolyzed by hog kidney (1-3) and other tissue preparations (4-7). The question is whether these substrates are hydrolyzed by the same enzyme and whether this or a different enzyme is responsible for the hydrolysis of free, unsubstituted dipeptides. In order to answer this question, we have attempted to separate and distinguish by various criteria the enzymatic activities in certain tissue homogenates toward chloroacetylalanine and glycylalanine on the one hand, and chloroacetylalanine and chloroacetylalanylglycine on the other. As supplemental to the study on the separation of the activities toward chloroacetylalanine and glycylalanine, a similar study was made on the separation of the activities toward the analogous unsaturated substrates, chloroacetyldehydroalanine and glycyldehydroalanine.

The experimental procedures employed included (a) determinations of the susceptibility of the various substrates at different pH values, (b) differential heat inactivation, (c) differential alcohol inactivation, (d) alcohol-acid fractionation of the tissue proteins at low temperature, and (e) distribution of activities between sediment and supernatant after high speed centrifugation of the homogenate.

## EXPERIMENTAL<sup>1</sup>

*Relation of Activity to pH*—The hydrolysis of chloroacetyl-DL-alanine by hog kidney homogenates possesses an optimum at pH 7.0 (3).<sup>2</sup> Under

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<sup>1</sup> The hydrolysis of the saturated substrates was followed by the ninhydrin-CO<sub>2</sub> manometric method. Activities are represented in terms of micromoles of substrate (L form of the alanine residue) hydrolyzed per hour per mg. of N of enzyme preparation, and are determined from the linear region of the hydrolysis-time curves up to 30 per cent hydrolysis of the substrate. The hydrolysis of the dehydropeptides was followed by determinations of the evolved ammonia.

<sup>2</sup> The shape of the pH-activity curve in hog kidney homogenates for chloroacetylphenylalanine is practically identical with that for chloroacetylalanine, with an optimal susceptibility for both substrates at pH 7.0. Using carbobenzoxyglycyl-L-

the same conditions, the hydrolysis of chloroacetyl-glycyl-DL-alanine, chloroacetyl-DL-alanylglycine, and glycyl-DL-alanine possesses an optimum at pH 8.0 (3). With homogenates of rat spleen, the optimal pH for the hydrolysis of chloroacetyl-L-alanine is at 6.0, whereas for glycyl-L-alanine it is at 7.6 (Fig. 1).

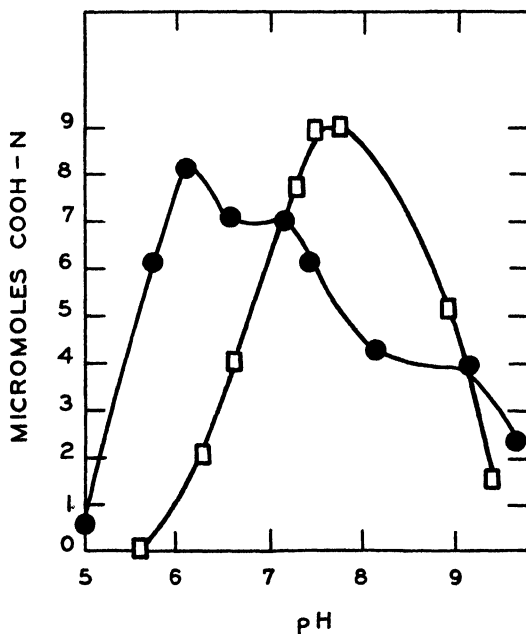


FIG. 1. Hydrolysis at different pH values of chloroacetyl-L-alanine (●) and glycyl-L-alanine (□) by rat spleen homogenates. The digests consisted of 1 cc. of diluted aqueous homogenate, 1 cc. of buffer solution, and 1 cc. of either water or 0.025 M neutralized substrate. From pH 5 to 8 a 0.066 M phosphate buffer was used; above pH 8, a 0.10 M borate buffer was used. For chloroacetylalanine the tissue preparation contained 2 mg. of N per cc.; for glycylalanine the tissue preparation contained 0.1 mg. of N per cc. Temperature 38°.

*Differential Heat Inactivation*—These experiments were conducted with homogenates of hog kidney in glycerol which were observed to be inactivated much more slowly and evenly by heat than the corresponding aqueous homogenates. The homogenates were prepared by mixing 1 part of the tissue with 9 parts of 90 per cent glycerol in a Waring blender and

phenylalanine and carbobenzoxyglycyl-L-tyrosine as substrates, Bergmann and Fruton reported the presence of a carboxypeptidase in hog kidney preparations with an optimal activity at pH 5.4 (5). At this pH, the susceptibility of chloroacetyl-L-phenylalanine is nearly zero. Results with racemic substrates were identical with those obtained with the corresponding L forms.

allowing the mixture to stand for 16 hours at 30°. The mixture was then lightly centrifuged and stored at 5°. The activity of these preparations in glycerol toward several substrates was only slightly less than that of comparable aqueous homogenates of the same total N content.

Heated preparations were made by subjecting the undiluted glycerol extracts to a temperature of 60° for 25 minutes. The effect of this heating is noted in the data of Fig. 2. Whereas the decrease in the activity toward glycyl-L-alanine was considerable, the decrease in the activity toward

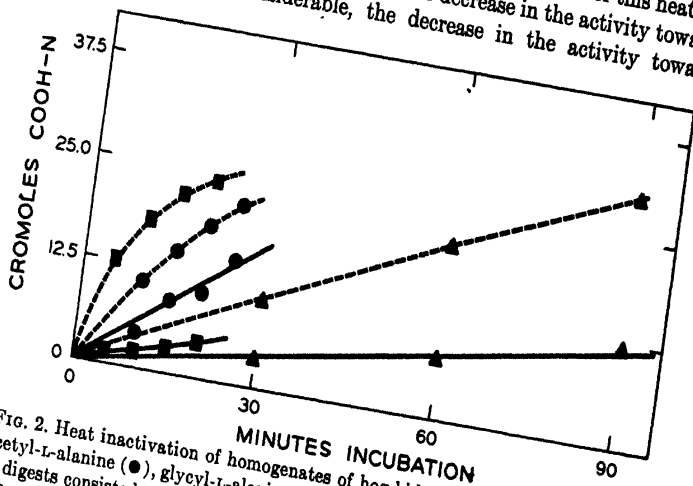


Fig. 2. Heat inactivation of homogenates of hog kidney in glycerol toward chloroacetyl-L-alanine (●), glycyl-L-alanine (■), and chloroacetyl-DL-alanylglycine (▲). The digests consisted of 1 cc. of heated or unheated preparation in glycerol, 1 cc. of 0.066 M phosphate buffer at the appropriate optimal pH, and 1 cc. of either water or 0.025 M substrate based upon a single optical form. For the above substrate, the tissue preparation concentrations were 0.118 mg., 0.059 mg., and 1.1 mg. of N per cc. respectively. Temperature of incubation was 38°. The solid curves represent heated homogenates.

chloroacetyl-L-alanine and toward chloroacetyl-DL-alanylglycine was much smaller in magnitude.\*

**Differential Alcohol Inactivation**—In contrast to the results of the experiments on heat inactivation, the effect of addition of alcohol to 10 per cent causes a considerably greater inactivation of aqueous hog kidney homogenates toward chloroacetyl-L-alanine than toward glycyl-L-alanine (Fig. 3).

**Fractionation Studies**—A separation of the proteins in the hog kidney homogenates was achieved by increasing the alcohol concentration, by low-

\* A separation by differential heat inactivation of the activities toward dipeptides and acylated amino acids by procaryboxypeptidase has been reported (8) (cf. (5)).



ering the pH through addition of acetic acid, and by decreasing the storage temperature from  $+2^{\circ}$  to  $-15^{\circ}$ . The centrifugation of the protein fractions was generally performed at the storage temperatures. The alcoholic supernatants were diluted with water until the alcohol content was less than 0.2 per cent before they were tested for activity. The sediments

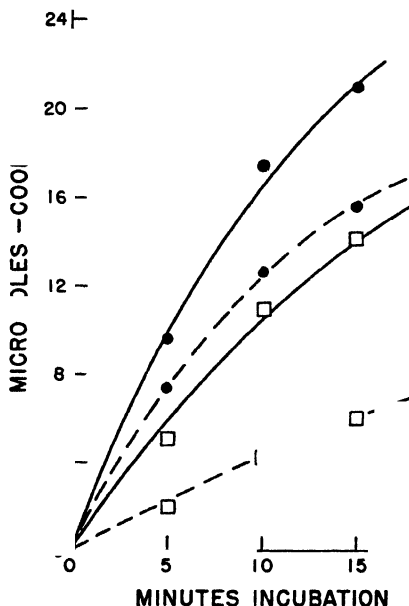


FIG. 3. Alcohol inactivation of hog kidney homogenates toward chloroacetyl-L-alanine (□) and toward glycyl-L-alanine (●). The digests consisted of 1 cc. of homogenate diluted with 0.3 cc. of either water or absolute alcohol, 1 cc. of 0.066 M phosphate buffer at optimal pH for each substrate, and 1 cc. of either water or 0.025 M neutralized substrate. For the above substrates, the tissue preparations contained 0.044 mg. of N per cc. Temperature  $38^{\circ}$ . The broken curves represent alcohol-treated homogenates.

after centrifugation were dialyzed against distilled water to remove the last traces of alcohol.

*Separation of Activities toward Chloroacetyl-L-alanine and Glycyl-L-alanine*—1 part of decorticated hog kidneys was mixed with 2 parts of distilled water in a Waring blender. The homogenate was then centrifuged at  $0^{\circ}$  and at 2800 R.P.M. in an International refrigerated centrifuge for 1 hour and the supernatant labeled Sc. The supernatant was then brought to pH 5.7 and ethanol added to bring the final concentration to 15 per cent. The mixture was stored at  $-3.5^{\circ}$  for 16 hours. The sediment ( $P_1$ ) was then centrifuged at  $-3.5^{\circ}$ . To the supernatant ( $S_1$ ) was added

alcohol to 30 per cent and the mixture was allowed to stand for 18 hours at  $-15^{\circ}$ . The sediment was centrifuged at this temperature and both the supernatant ( $S_2$ ) and the sediment ( $P_2$ ) examined for activity. The data are given in Table I. In alcohol-water mixtures of the composition employed the activity toward chloroacetylalanine is more soluble than that toward glycyl-L-alanine, and a clear cut separation can be achieved.

The recovery of the respective enzymatic activities in the various fractions is reasonably good. The total activity on chloroacetylalanine

TABLE I

*Separation of Activities toward Chloroacetyl-L-alanine and Glycyl-L-alanine in Hog Kidney Homogenates\**

Fraction	Substrates				Ratio
	Chloroacetylalanine†		Glycylalanine‡		
	Activity	Total activity§	Activity	Total activity§	
Supernatant Sc . . . . .	1036	1,744,620	2826	4,758,980	0.4
Sediment P <sub>1</sub> . . . . .	302	384,140	2181	2,774,230	0.1
Supernatant S <sub>1</sub> . . . . .	2964	1,224,132	665	274,640	4.4
Sediment P <sub>2</sub> . . . . .	2150	111,800	1043	54,170	2.1
Supernatant S <sub>2</sub> . . . . .	1881	289,670	369	56,820	5.1

\* Activities in terms of micromoles of substrate hydrolyzed per hour per mg. of N at  $37^{\circ}$ . The digests consisted of 1 cc. of enzyme solution, 1 cc. of phosphate buffer at optimal pH, and 1 cc. of either water or 0.025 M neutralized substrate solution.

† Activity in original homogenate, 800.

‡ Activity in original homogenate, 2260.

§ Total activity in terms of micromoles of substrate hydrolyzed per hour by entire volume of enzyme preparation (activity  $\times$  total N).

in  $P_1 + S_1$  gives a value of 1,608,270 as compared with 1,744,620 for the original supernatant  $S_c$ , or 92 per cent. Values for  $P_2 + S_2$  yield 401,470, as compared with a total activity of 602,200 on chloroacetylalanine in the amount of  $S_1$  taken for the further fractionation, or 67 per cent.

The total activity on glycylalanine in  $P_1 + S_1$  gives a value of 3,048,870 as compared with 4,758,980 for the original supernatant  $S_c$ , or 65 per cent. The amount of  $S_1$  taken for subsequent fractionation had a total activity of 137,320. Values for  $P_2 + S_2$  derived from this fraction had a total activity of 110,990, or 81 per cent.

*Separation of Activities toward Chloroacetyl-DL-alanine and Chloroacetyl-DL-alanylglycine*<sup>4</sup>—Decorticated hog kidneys were blended as above with

<sup>4</sup> Racemic and optically active substrates may be used interchangeably in these experiments, for, under the conditions used, the susceptibility of the L form which is measured is practically independent of the presence of the D form.

water, centrifuged, the supernatant (Sc) brought to pH 6.3, and alcohol added to 15 per cent. The sediment after centrifugation was discarded, and to the supernatant (S<sub>1</sub>), adjusted again to pH 6.3, alcohol was added to 30 per cent and the temperature lowered to  $-10^{\circ}$ . After standing for 16 hours at this temperature, the mixture was centrifuged at  $-10^{\circ}$  and the sediment again discarded. The pH of the supernatant (S<sub>2</sub>) was brought to 5.7, the mixture allowed to stand at  $-10^{\circ}$  for 5 hours, and the sediment (P<sub>3</sub>) removed by centrifugation. This sediment was dialyzed and brought to pH 5.5, and alcohol was added at  $-15^{\circ}$  to 30 per cent. After 5 hours, the sediment (P<sub>4</sub>) was centrifuged clear and dialyzed. Repetition of these procedures with alcohol to 15 per cent yielded sediments P<sub>5</sub> and

TABLE II  
*Separation of Activities toward Chloroacetyl-DL-alanine and  
Chloroacetyl-DL-alanylglycine\**

Fraction	Substrates		Ratio
	Chloroacetyl- alanine†	Chloroacetyl- alanylglycine‡	
Supernatant Sc .. . . .	912	22	41
Sediment P <sub>3</sub> .. . . .	3432	58	59
" P <sub>4</sub> .. . . .	3332	75	44
" P <sub>5</sub> .. . . .	2950	187	16
" P <sub>6</sub> .. . . .	700	165	4

\* The activities are the same as in Table I.

† Activity in original homogenate, 800.

‡ Activity in original homogenate, 25. The distribution of activity toward chloroacetylglucyl-DL-alanine in each of the fractions was very nearly the same as that given above for chloroacetyl-DL-alanylglycine. The latter substrate is hydrolyzed nearly 3 times faster than the former.

P<sub>6</sub> consecutively. Further alcohol treatment produced such marked reduction in the activities toward both substrates as to render the results dubious. The data on the fractions obtained to P<sub>6</sub> are given in Table II.

The marked drop in activity of P<sub>6</sub> toward chloroacetylalanine might have been due to alcohol inactivation to which the enzyme, active on this substrate, is quite susceptible (see the section on "Differential alcohol inactivation"). If this lowered value for P<sub>6</sub> were due to incomplete precipitation, the value for the supernatant should show a corresponding rise in activity; the activity, however, in this supernatant was too low and the alcohol concentration too high to be able to measure the former conveniently. Total activities could therefore not be calculated.

*Distribution of Activities toward Chloroacetyl-DL-Alanine and Chloroacetyl-DL-alanylglycine between Sediment and Supernatant*—Fresh hog kidney

cortex was homogenized in the 5-fold weight of cold distilled water and the homogenate subjected to centrifugation at  $9000 \times g$  for 20 minutes at  $0^\circ$ . The heavy particles so removed possessed relatively little activity toward either substrate. The supernatant Sc was then centrifuged at  $26,000 \times g$  for 2 hours at  $0^\circ$ , and the activities in the supernatant ( $S_1$ ) and the sediment ( $P_1$ ) compared. The data are given in Table III. The recovery of total activity from sediment plus supernatant on chloroacetylalanine is 78 per cent, that on chloroacetylalanylglycine 69 per cent of the original.

From two lines of evidence, the activities toward chloroacetylalanine and chloroacetylalanylglycine have thus been distinguished (a) by progressive precipitation with alcohol, whereby the enzyme which hydrolyzes the acylated dipeptide becomes increasingly less soluble (Table II), and

TABLE III  
*Distribution of Activities toward Chloroacetyl-DL-alanine and  
Chloroacetyl-DL-alanylglycine between Supernatant and  
Sediment in Aqueous Homogenates\**

Fraction	Substrates				Ratio
	Chloroacetylalanine		Chloroacetylalanylglycine		
	Activity	Total activity	Activity	Total activity	
Supernatant Sc . . . . .	785	218,520	25	6800	31
“ S <sub>1</sub> . . . . .	1300	159,900	20	2460	65
Sediment P <sub>1</sub> . . . . .	280	9,800	64	2240	4

\* Activities and total activities are the same as in Table I.

(b) by high speed centrifugation of the aqueous kidney homogenate, whereby the activity toward chloroacetylalanylglycine is found in greater measure in the sediment and that toward chloroacetylalanine in greater measure in the supernatant (Table III).

*Separation of Activities toward Chloroacetyldehydroalanine and Glycyldehydroalanine*—The enzymes in kidney responsible for the hydrolysis of chloroacetyl-L-alanine and of glycyl-L-alanine are water-soluble. So too is the enzyme which hydrolyzes chloroacetyldehydroalanine, but that responsible for the activity toward glycyldehydroalanine is largely associated with the insoluble particulate matter of this tissue.<sup>5</sup> We have noted in agreement with Shack that these enzymes may be readily separated by the following procedure. Hog kidneys were mixed with 5 times their weight of distilled water in a Waring blender. The homogenate was centrifuged for 20 minutes at  $9000 \times g$  at  $0^\circ$  and the relatively inactive

<sup>5</sup> Personal communication from Dr. J. Shack.

sediment discarded. The supernatant hydrolyzed chloroacetyldehydroalanine with an activity rate of 50  $\mu\text{M}$  per hour per mg. of N and glycyldehydroalanine with a corresponding activity of 550 (ratio 0.1). The total activity toward these substrates was 8000 and 84,000  $\mu\text{M}$  per hour, respectively. When this supernatant was centrifuged at  $0^\circ$  at  $26,000 \times g$  for 1 hour, a sediment was obtained which hydrolyzed chloroacetyldehydroalanine at a rate of 9 (total activity 280) and glycyldehydroalanine at a rate of 1005 (total activity 31,000) (ratio 0.009), and a supernatant which hydrolyzed these substrates at rates, respectively, of 45 (total activity 6210) and 169 (total activity 23,320) (ratio 0.3). The total activity toward chloroacetyldehydroalanine in sediment and supernatant, 6490, is thus 81 per cent of the original, and that toward glycyldehydroalanine, namely 54,320, is 65 per cent of the original. The greatly different shapes of the pH-activity curves for these substrates are another means of distinguishing the enzymatic activities responsible for their hydrolysis (3, 9, 10).

Thus not only are chloroacetyl-L-alanine and glycyl-L-alanine hydrolyzed by two different enzymes, but so also are chloroacetyldehydroalanine and glycyldehydroalanine. The enzymes active on the saturated substrates are both relatively water-soluble in kidney homogenates, and are not readily sedimentable after centrifugation at  $26,000 \times g$  for 1 hour (*cf.* (3)). In alcohol-water mixtures, however, that enzyme responsible for the hydrolysis of chloroacetylalanine is the more soluble (Table I). The separation in aqueous kidney homogenates of the activities toward the corresponding unsaturated substrates is more easily accomplished, for the activity toward chloroacetyldehydroalanine is largely soluble at  $26,000 \times g$ , whereas the activity toward glycyldehydroalanine, although partly soluble, is largely insoluble. By this criterion, the enzymes active on glycyl-L-alanine and glycyldehydroalanine are also readily separated (*cf.* (11)).<sup>6</sup> Thus far, the activities toward chloroacetyl-L-alanine and chloroacetyldehydroalanine have not been clearly separated. It appears probable that the chloroacetylated dipeptides are attacked by enzymes different from those which attack the chloroacetylated amino acids, the former being associated with larger, more readily sedimentable particles; the same distinction possibly holds for the enzymes acting on the corresponding unsaturated compounds (*cf.* (3)). The primary point of enzymatic attack on the acylated dipeptides is not yet known (3). Since only the initial hydrolysis rate of chloroacetylalanylglycine is determined, presumably only one of the two available bonds of the L isomer of this tripeptide was attacked (*cf.* (3)).

*Attempts to Separate Activities toward Acetyl-L-alanine, Chloroacetyl-L-*

<sup>6</sup> The D-peptidase acting upon glycyl-D-alanine is also sedimentable at  $26,000 \times g$  (11), and can thus be distinguished from the more soluble L-peptidase.

*alanine, and Chloroacetyl-L-phenylalanine*—No way of distinguishing the activities toward these three compounds could be found by any of the procedures described above. Thus, by the alcohol fractionation procedure no changes in activity ratios could be detected in any fraction tested for these compounds, with either hog kidney or rat liver as the enzyme source.<sup>7</sup> Chloroacetylalanine is readily hydrolyzed by kidney preparations, whereas chloroacetylphenylalanine is only weakly attacked (1). The reverse is true of pancreas preparations: The kidney enzyme is related to the carboxypeptidase of the pancreas in so far as its specificity appears to be restricted to peptides lacking a free  $\alpha$ -amino group (non-ampholytes). However, the relative action of kidney and of pancreas on chloroacetylalanine and chloroacetylphenylalanine strongly indicates that two quite different systems are involved in the two tissues. The presence of a  $\beta$ -phenyl group enhances susceptibility of the substrate to the action of the pancreas enzyme, whereas it diminishes susceptibility of the substrate to the action of the kidney enzyme. Neither kidney nor pancreas enzymes act upon the acylated D-amino acids.

Attempts to separate the activities toward acetylglycine and benzoylglycine in hog kidney homogenates by low temperature alcohol fractionation were inconclusive because of the relatively low activity of all fractions toward the latter substrate (0.6 in the original homogenate). The activity toward acetylglycine could be increased some 6-fold and that toward benzoylglycine some 15-fold, but, in view of the relatively small changes in activity ratios on fractionation, as well as the low order of magnitude of activity toward benzoylglycine, we are reluctant to impute to these changes any clear cut separation.

#### SUMMARY

1. On the basis of the relation of activity to pH, of differential heat and alcohol inactivation, and of fractionation in alcohol-water mixtures at low temperatures, the enzymatic activities toward chloroacetyl-L-alanine and toward glycyl-L-alanine have been distinguished. The optimal activity of rat spleen homogenates toward the former substrate is at pH 6.0, toward the latter at pH 7.6. With hog kidney preparations, the heat stability of the activity toward glycyl-L-alanine is less than that toward chloroacetyl-L-alanine; the stability of the respective activities is reversed when the fresh preparation is treated with alcohol to 10 per cent. In alcohol-water

<sup>7</sup> Preliminary studies revealed that addition of cysteine caused about a 25 per cent inhibition in the capacity of hog kidney preparation P<sub>2</sub> (Table II) to hydrolyze chloroacetyl-L-alanine, and little or no effect on the hydrolysis of chloroacetyl-L-phenylalanine. On the other hand, cobalt salts added up to 0.012 M accelerated the hydrolysis of chloroacetyl-L-phenylalanine to an apparently greater extent than that of chloroacetyl-L-alanine.

mixtures the activity toward chloroacetylalanine is the more soluble and a clear cut separation from that toward glycyl-L-alanine can be made.

2. The activities toward chloroacetyldehydroalanine and glycyldehydroalanine can be distinguished by a single high speed centrifugation of aqueous hog kidney homogenate, that toward the former substrate remaining largely in the supernatant and that toward the latter substrate precipitating mostly in the sediment.

3. On the basis of alcohol fractionation at low temperature and of high speed centrifugation of the homogenate, the activities in hog kidney toward chloroacetylalanine and chloroacetylalanylglycine have been distinguished. In alcohol-water mixtures, as well as under conditions of high speed centrifugation of the aqueous homogenate, the activity toward the latter substrate is the less soluble.

4. The methods employed were unable to distinguish the activities in hog kidney toward acetylalanine, chloroacetylalanine, and chloroacetylphenylalanine.

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# THE RELATION OF AMINO ACID AVAILABILITY IN DIETARY PROTEIN TO LIVER ENZYME ACTIVITY\*

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In 1948 Miller (1) demonstrated that the loss of liver xanthine oxidase activity in the rat greatly exceeded the loss of liver protein during acute inanition. Moreover, xanthine oxidase appeared to be the most labile of the four liver enzymes studied. Westerfeld and Richert (2) observed that increasing the level of protein in the diet tended to bring about an increase in liver xanthine oxidase activity, although there was no *direct* correlation between the level of dietary protein and enzyme activity.

Because of the apparent lability of liver xanthine oxidase, the present work was undertaken to observe whether the measured activity of this enzyme could be used as an index of general protein metabolism. In this work the question of availability of amino acids in dietary protein has been related to liver xanthine oxidase activity under conditions in which gross body changes are not in general sensitive enough to reflect small protein variations in the animal body. It has been found that, at a level of whole dietary protein which maintains and even supports growth of the adult rat, liver xanthine oxidase activity may be appreciably decreased, probably because of incomplete assimilation of the amino acids in the protein.

Early in the experiments it was observed that animals fed a 14.6 per cent casein diet exhibited much lower liver xanthine oxidase activity than rats fed a good stock ration. It was also observed that if the animals were fed acid-hydrolyzed casein at an 18 per cent level (isonitrogenous with 14.6 per cent casein) the xanthine oxidase activity based on liver protein was also much higher than that in animals fed the 14.6 per cent casein diet. If the animals were unable to digest completely the ingested whole protein, the poor availability of one or more essential amino acids could account for the low xanthine oxidase activity. On the other hand if the protein were fed in a predigested form, *i.e.*, as acid-hydrolyzed casein, this difficulty should not be encountered and xanthine oxidase activity should be normal. To rule out the possibility of the formation of unknown factors

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during acid hydrolysis of casein which might in some way increase liver xanthine oxidase activity, a mixture of purified amino acids simulating casein was fed and xanthine oxidase activity of the livers of the animals determined.

If the animals were unable to obtain enough of one or more amino acid from a 14.6 per cent casein diet, it was possible that feeding a higher level of casein might furnish the required amino acid levels by a mass action effect. Therefore, other experiments were carried out in which the animals were given a 40 per cent casein diet.

From calculation of the essential amino acid concentrations in a 14.6 per cent casein diet, methionine was found to be slightly below the level stated by Womack and Rose (3) to be necessary for growth of young, growing rats. Although we used adult animals in our experiments, it was decided to increase the methionine level in the 14.6 per cent casein ration to a value slightly above that required for the young rat. Therefore 0.25 per cent extra methionine was added to the ration and xanthine oxidase activity determined in the livers of the animals fed this ration.

#### EXPERIMENTAL

Adult, male albino rats of the Holtzman strain weighing 250 to 350 gm. were maintained on a good stock ration for 2 weeks before being placed on the purified rations.

A series of five synthetic rations was prepared, similar in all respects except for the type or quantity of protein included. The rations contained the following common components: Salts IV (4) 4 gm., corn oil 5 gm., vitamin mixture<sup>1</sup> 2 gm., protein at the desired level, and sucrose to make 100 gm. In addition, 2 drops of halibut liver oil were administered each week by dropper. The protein contents of the five rations were as follows: Ration I, 14.6 per cent Smaco casein; Ration II, 14.35 per cent Smaco casein + 0.25 per cent DL-methionine; Ration III, 18 per cent acid-hydrolyzed casein (5) + 0.5 per cent DL-tryptophan; Ration IV, 24.7 per cent purified amino acid mixture<sup>2</sup> corresponding to casein (6); and Ration V, 40 per cent Smaco casein. The acid-hydrolyzed casein was

<sup>1</sup> 100 gm. of vitamin mixture contained the following vitamins in a sucrose base: thiamine hydrochloride 10 mg., riboflavin 15 mg., niacin 75 mg., pyridoxine 12.5 mg., calcium pantothenate 100 mg., biotin 0.5 mg., pteroylglutamic acid 1 mg., choline chloride 5 gm., and *D*-inositol 0.5 gm.

<sup>2</sup> 24.7 gm. of amino acid mixture contained the following amounts of purified amino acids: DL-alanine 0.90, L-glutamic acid 3.7, L-cystine 0.06, DL-leucine 3.92, DL-phenylalanine 0.84, DL-valine 2.26, DL-aspartic acid 1.0; glycine 0.8, L-histidine hydrochloride 0.50, DL-isoleucine 2.10, L-lysine hydrochloride 2.80, L-proline 1.33, L-tyrosine 1.0, DL-methionine 0.57, DL-threonine 1.26, L-arginine hydrochloride 0.45, DL-serine 1.21, DL-tryptophan 0.29 gm.

analyzed microbiologically for arginine, glutamic acid, and the essential amino acids. The amino acids shown to be decreased by the acid hydrolysis from reported casein levels were supplemented with purified amino acids to the required levels. Since tryptophan was completely destroyed during acid hydrolysis, 0.5 per cent DL-tryptophan was added to bring the L-tryptophan level up to that of casein. When the amino acid mixture in Ration IV was fed at a 24.7 per cent level, the concentrations of amino acids utilizable by the rat were equivalent to those found in 14.6 per cent casein, since only the L forms of leucine, valine, isoleucine, lysine, and threonine are active for the rat (7).

The animals were maintained on the respective rations for at least 2 weeks before being used in the enzyme studies. Six animals were maintained on each ration, except that eight animals comprised the group receiving Ration III.

After the feeding period, the animals were stunned by a blow on the head, decapitated, and exsanguinated. The livers were removed *in toto*, placed immediately into cracked ice, and chilled for several minutes. They were then blotted free of moisture and weighed. A portion of each liver was homogenized in 5 volumes of ice-cold 0.039 M sodium potassium phosphate buffer (pH 7.3), and xanthine oxidase activity was determined according to the method of Axelrod and Elvehjem (8) with a Warburg bath maintained at 30°.

Total nitrogen, non-protein nitrogen, and dry weight of aliquots of each homogenate were determined in duplicate or triplicate. All nitrogen analyses were made by a modification of the micro-Kjeldahl technique. Non-protein N was determined by precipitating the protein in 5 ml. aliquots of the homogenates with 20 ml. of 20 per cent trichloroacetic acid, heating for 2 minutes at 100°, and filtering. The determination of nitrogen in 10 ml. aliquots of the filtrate gave the non-protein N of the liver homogenates. Homogenate protein was calculated from total N less non-protein N per gm. of liver times the protein factor 6.25. To serve as a check on how accurately the liver homogenates were prepared, total N of approximately 0.1 gm. portions of each liver was determined.

The results of xanthine oxidase activity were calculated in terms of liver protein, dry weight, wet weight, and activity per 100 gm. of rat. In this way the relation of xanthine oxidase activity to a variety of liver factors, *e.g.*, total liver protein, liver solids, liver moisture, and body weight, could be obtained.

#### RESULTS AND DISCUSSION

The results of the enzyme determinations for the various groups of animals are presented in Table I. Although the standard errors of the mean

are given only for enzyme activity based on liver protein, approximately the same relative deviations hold for the other results.

When xanthine oxidase activity was based on liver protein in the animals receiving the different diets, it was observed that, although the animals were gaining weight in every case, the liver xanthine oxidase activity of the group receiving 14.6 per cent casein was much lower than that of any other group. When 0.25 per cent methionine was added to the 14.6 per cent casein ration, liver xanthine oxidase activity returned nearly to normal. Moreover, when the protein was either acid-hydrolyzed casein or the purified amino acid mixture, xanthine oxidase activity based

TABLE I  
*Relation of Liver Xanthine Oxidase Activity in Rat to Dietary Protein*

Ration*	Activity per gm. liver protein	Activity per gm. dry liver	Activity per gm. wet liver	Activity per 100 gm. rat	Average gain in weight per week
	$\mu\text{l. O}_2 \text{ per hr.}$	$\mu\text{l. O}_2 \text{ per hr.}$	$\mu\text{l. O}_2 \text{ per hr.}$	$\mu\text{l. O}_2 \text{ per hr.}$	gm.
I	590 $\pm$ 40	360	99	350	+2
II	1080 $\pm$ 60	680	163	790	+18†
III	900 $\pm$ 100	540	100	490	+3
IV	1100 $\pm$ 90	630	180	630	+3
V	1200 $\pm$ 30	670	182	790	+9
Stock	1200 $\pm$ 150	680	190	720	+8

\* See the text for composition.

† These animals were somewhat younger than the other animals when they were placed on the purified ration. They were maintained on the purified ration, however, until their average weight was the same as that of the animals in the other groups. This probably accounts for the large value here, since these figures were calculated from weight changes throughout the period of feeding the purified diets.

on liver protein was also nearly normal. It was concluded from these results that methionine was probably the limiting amino acid in the 14.6 per cent casein ration. The fact that the level of cystine is so low in whole casein makes it appear doubtful that it was important in this problem. In the acid-hydrolyzed casein ration cystine was probably still lower than in the casein ration because of destruction during the hydrolysis. No extra cystine was added to the acid-hydrolyzed casein ration, however, because of lack of adequate methods for assaying for that amino acid in the hydrolysate. It appeared also that, although there was enough methionine in a 14.6 per cent casein ration to keep xanthine oxidase activity at a normal level, the animals were unable to utilize all the methionine present in the whole protein. This lack of utilization was possibly due to incomplete digestion of the protein, since the same level of methionine in either

acid-hydrolyzed casein or the purified amino acid mixture was enough to keep xanthine oxidase at nearly a normal level.

It is interesting to observe in these experiments that the enzyme activity based on liver protein followed very closely the pattern observed when activity was based on dry weight. However, a somewhat different pattern was observed when enzyme activity was based on wet weight of the liver. The enzyme results based on wet weight of the liver for the two groups of animals receiving 14.6 per cent casein and acid-hydrolyzed casein were nearly the same and also lower than those for the other groups. The reasons for these results are not clear, although they may possibly be explained by a higher salt content of the acid-hydrolyzed casein. This would tend to increase the water intake, and consequently the water content of the liver, with a concomitant decrease in wet weight enzyme ac-

TABLE II  
*Relation of Liver Nitrogen and Non-Protein Nitrogen in Rat to Dietary Protein*

Ration*	Nitrogen per gm. liver	Non-protein N per gm. liver
I	0.032	0.0032
II	0.030	0.0032
III	0.030	0.0033
IV	0.030	0.0035
V	0.033	0.0032
Stock	0.031	0.0032

\* See the text for composition.

tivity. The possibility of the presence of factors toxic to the rat in acid-hydrolyzed casein also should not be overlooked.

When the results were expressed as enzyme activity per 100 gm. of rat, the pattern most nearly approximated that observed when the results were based upon liver dry weight. The differences in enzyme activity between the groups of animals were not greatly amplified, as sometimes occurs when enzyme results are expressed in this manner. This was probably due to the fact that the weights of the animals of all groups were approximately the same when used in the enzyme studies.

In Table II the results of the total N and non-protein N determinations for the various groups of animals are presented. The total N results were obtained from the nitrogen determinations upon portions of the whole livers. Very few if any significant differences were observed either in total N or non-protein N among the different groups of animals. Other workers (9) have reported measurable decreases in liver protein per gm. of liver under severe conditions of protein depletion. It thus appears

that xanthine oxidase activity may decrease markedly without a noticeable decrease in non-enzyme liver protein.

The changes in xanthine oxidase activity observed in these experiments were probably due to an actual decrease in enzyme protein rather than to variations in dietary riboflavin (10), since all groups of animals received the same adequate level of riboflavin throughout the feeding period. Similarly, although pteroylglutamic acid has been shown to influence xanthine oxidase activity profoundly both *in vitro* (11) and *in vivo* (12), the effects we have observed in the present experiments cannot be attributed directly to that factor.

#### SUMMARY

1. Evidence has been presented that liver xanthine oxidase activity can be used as a sensitive index of amino acid availability in dietary proteins.

2. With use of liver xanthine oxidase activity as a criterion, it has been demonstrated that methionine in dietary casein is not readily available to the rat. However, if either acid-hydrolyzed casein or a mixture of purified amino acids simulating casein is fed as the source of protein, the rat appears to utilize the ingested amino acids much more completely.

3. The effects of the low availability of methionine in casein fed at the 14.6 per cent level upon liver xanthine oxidase can be overcome by feeding a 40 per cent casein diet.

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# FACTORS INFLUENCING GALACTOSE UTILIZATION\*

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A relationship between fat and the utilization of galactose was first reported by Schantz *et al.* in 1938 (1). They fed mineralized skim milk to rats and found as much as 35 per cent of the ingested galactose in the urine. When whole milk was fed or when skim milk was supplemented with one of several other fats at the level of 4 per cent, galactosuria was prevented. This observation was confirmed by Geyer *et al.* (2) who extended the work to include synthetic diets containing lactose or galactose. Nieft and Deuel (3) found that increased levels of fat resulted in lower galactosuria. They also found that the rate of intestinal absorption of galactose varies inversely with the percentage of fat in the diet.

On the other hand Zialcita and Mitchell (4) fed a lactose-casein-salts diet to which they added butter fat, corn oil, or glucose in isocaloric amounts, and found approximately equal galactose losses in the urine. They concluded that fat as such does not favor galactose utilization in the animal.

The possibility was considered that these conflicting results might have been due to inadequate control of galactose intake per unit of body weight. Therefore, studies were initiated in which the galactose intake was controlled by administering galactose solutions to rats through a stomach tube, while permitting them to eat fat-free or fat-containing diets *ad libitum*.

*Method for Galactose Determinations*—Total sugar, or more correctly total reducing substances, in the urine were determined colorimetrically by a micromethod of Somogyi (5) with Nelson's arsenomolybdate color reagent (6). The boiling period was extended to 30 minutes for more complete oxidation of the galactose. The urine samples were diluted so that 1 ml. would contain the equivalent of 15 to 50  $\gamma$  of galactose. 1 and 2 ml. samples, each in duplicate, were used in all determinations.

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Samples of standard galactose solutions were included with every assay. For the galactose determinations 9 ml. samples of the above dilution were placed in a centrifuge tube, to which was added 0.2 ml. of 50 per cent yeast suspension (1 gm. of bakers' yeast after six washings was made to 2 ml. and stored below freezing temperature). After incubation at 37° for 30 minutes, the yeast cells were centrifuged down. 1 and 2 ml. samples were removed and carried through the procedure outlined above. Special attention was paid in neutralizing the samples before the addition of the copper reagent, because it was found that the yeast treatment lowered the pH, and in this method it is important to keep the pH between 7 and 9.

It should be mentioned that, in the galactose determination, other non-fermentable reducing substances which might be present are also measured as galactose. The average daily excretion of such substances by rats on a lactose- and galactose-free diet was found to be 15.7 mg. per 100 gm. of body weight. In each case this figure was subtracted in order to obtain the true galactose value. All values are expressed on the basis of 100 gm. of body weight.

*Studies with Purified Diets*—Rats of the Sprague-Dawley strain weighing about 120 gm. were used in these experiments. They were housed individually in metabolism cages, food consumption records were kept, and the urine was collected under toluene. Each experiment included three groups of rats which were given the following basal diets (the figures represent parts).

	Diet 1	Diet 2	Diet 3
Casein.....	2.0	6.5	2.0
Salts IV (7).....	0.4	0.4	0.4
Butter fat.....	2.0		
Sucrose.....			4.5

2 drops of haliver oil per rat per week were the source of the fat-soluble vitamins. Water-soluble vitamins were mixed into the basal ration.<sup>1</sup> It will be noticed that all basal diets contained casein and Salts IV. The balance was made up by isocaloric amounts of either butter fat, casein, or sucrose.

In the first series, which included three experiments, different measured amounts of galactose were administered by stomach tube in three equal portions each day for 3 or 4 days. The basal diets constituted the part of the daily rations given *ad libitum*.

<sup>1</sup> Each 69 gm. of Diet 1 and each 100 gm. of Diets 2 and 3 were supplemented with 0.5 mg. of thiamine, 0.7 mg. of riboflavin, 0.6 mg. of pyridoxine, 3.0 mg. of calcium pantothenate, and 150 mg. of choline hydrochloride.

It was observed that the galactose excretion was proportional to the galactose intake per unit body weight (Fig. 1). Somewhat lower galactose excretion was obtained on Diet 1 (fat) especially at the lower galactose intakes. The galactosuria ranged from 30 to 70 per cent of the ingested galactose. It was suspected that these high galactose losses in the urine were due largely to the mode of galactose administration. Therefore in a

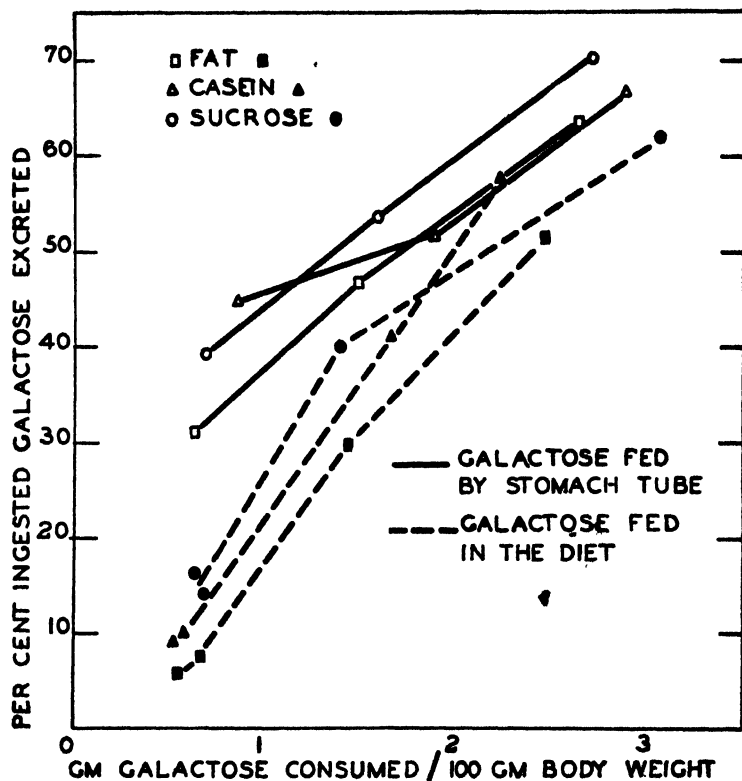


FIG. 1. Galactose excretion in the urine of rats on various levels of galactose intake by stomach tube and in their diets. Each point represents an average of three rats.

second series of experiments comparable amounts of galactose were incorporated in the diets which were consumed *ad libitum* and the amounts recorded. Four such experiments were conducted, each of which was of 3 days duration. Again it was observed that galactose excretion was proportional to the galactose intake. At low levels of galactose intake the galactose excretion was strikingly lower when the galactose was consumed in the diet, as compared to that excreted when the galactose was fed by stomach tube in three portions each day.



Since it was observed that the rate of administration of galactose exerted a definite effect on the percentage of galactose excreted it was considered advisable to study by another method the effect of delaying the availability of the galactose in the digestive tract.

This was done in a third series of experiments in which lactose was incorporated into the same diets replacing the galactose. Lactose was employed, since galactose would be slowly released by the hydrolysis in the tract. In the calculation of the results, the factor 180/342 was used to convert the amounts of lactose consumed into galactose. The results are shown in Table I. It is apparent that on the lactose diets considerably less galactose was excreted than when an equivalent amount of galactose

TABLE I  
*Galactose Excretion on Purified Diets Containing Lactose*

Diet No.	No. of days	No. of rats	Galactose ingested per 100 gm. body weight per day	Per cent ingested galactose excreted
			gm.	
1. Fat	2	3	0.83	0.28
	3	1	0.74	0.31
	2	3	0.41	0.60
2. Casein	2	3	0.73	2.67
	3	3	1.11	5.25
	2	3	0.51	1.78
3. Sucrose	2	3	0.87	1.93
	3	2	0.84	3.49
	2	3	0.46	1.01

was fed. When higher levels of lactose were mixed in the diets, severe diarrhea resulted, preventing accurate excretion data at high lactose consumptions.

*Studies with Milk Diets*—In early investigations on this problem, milk was fed and the galactose intake per unit of body weight was not considered. Therefore, attention was paid in the present studies to this factor. Three diets, whole milk, skim milk, and skim milk containing cerelose were used. In the third diet the cerelose was added in amounts isocaloric with the 4 per cent fat in the whole milk. In order to equalize the content of lactose in unit volume of the three diets, 96 ml. of skim milk were diluted to 100 ml. with water for the second diet; to another 96 ml. of skim milk 9 gm. of cerelose were added, and this was brought to 100 ml. for the third diet. In the calculation, 100 ml. of milk were considered to contain 4.78 gm. of lactose.

Rats weighing about 240 gm. each were divided into three groups and

placed on these diets *ad libitum* for 2 weeks of preliminary feeding. For the 2 day experimental periods, the rats were weighed daily and their respective milk diets were offered in four or five fresh portions each day, in amounts proportional to their body weight. Some rats on the second and third diets (skim milk and skim milk plus cerelese respectively) were restricted in their intake to equal that of the rats on whole milk, the latter having generally required less than the former two groups. Three other rats on the skim milk diet and two rats on the skim milk plus cerelese diet were allowed *ad libitum* amounts, which were likewise measured in 4 or 5 portions each day. The results are shown in Table II. It is observed that fat in the whole milk exerted a small but significant effect in lowering the galactosuria.

TABLE II  
*Galactose Excretion on Milk Diets*

Diet (2 days)	No. of rats	Galactose ingested per day per 100 gm. body weight	Per cent galactose excreted
		gm.	
Whole milk	4	0.634	0
	5	0.734	0
Skim "	6	0.640	0.83
	3	0.767	2.5
	3	1.017	1.3
" " + cerelese	5	0.635	0.7
	3	0.746	1.0
	2	0.902	1.75

#### DISCUSSION

These data indicate that one of the major factors controlling the excretion of galactose in the urine is the rate at which this sugar reaches the circulation. Observations leading to this conclusion are as follows: (a) When small amounts of galactose were given by stomach tube in three portions per day, a considerably larger fraction was excreted than when equal amounts were slowly ingested in the diet through the day (Fig. 1). At high levels of galactose intake the difference between the two methods of administration was smaller, because presumably the rate of absorption from the intestine was the limiting factor and not the amount of galactose present in the stomach and intestine at any one time. (b) Feeding higher amounts of galactose per unit time resulted in a much higher per cent excretion (Fig. 1). (c) When equivalent amounts of galactose were fed in the form of lactose, considerably less galactose was excreted (Fig. 1 and Table I), probably due to the gradual hydrolysis of lactose to galactose and glucose.

Coryell and Christman (8) found that, following the administration by stomach tube of 2 gm. of lactose per kilo of rat, an average of 32, 59, and 73 per cent of the sugar was hydrolyzed at the end of 1, 2, and 3 hours respectively. They further observed that glucose made available by the hydrolysis of lactose was absorbed as rapidly as it was formed, while the galactose was absorbed at a somewhat slower rate.

Our general observation that the galactose excretion in the urine is largely controlled by the rate at which this sugar reaches the circulation is in line with the conclusion of Dominguez and Pomerene (9) who found that the rate of galactose excretion is proportional to the plasma concentration. In their study they injected galactose intravenously into dogs and measured plasma concentration and urinary excretion of this sugar.

Concerning the rôle of fat in the utilization of galactose, the data presented show consistently some beneficial influence which becomes of greater importance at certain low levels of galactose intake or availability in the digestive tract. When the galactose intake is low enough, and particularly when it is gradually liberated from lactose, the galactose reaches the blood at such a slow rate that complete utilization follows. When this rate is exceeded and galactose appears in the urine, then the presence of fat appears to reduce or prevent the galactose excretion and consequently increases the utilization. At very high levels of galactose intake, the presence of fat appears to have less influence on the galactosuria. It is possible that the rate of absorption approaches its upper limit at these high levels. It is also possible that the rate of excretion becomes the limiting factor, but there is no evidence that fat has any influence on the excretion mechanism.

Since the effect of fat does not appear to be very great at any level of galactose intake, it would seem that the known rôles of fat in slowing gastric emptying and in delaying the absorption of galactose from the intestines (3) would completely account for the relatively small effect of fat on the galactose excretion.

In their study on the effect of the level of fat on the excretion of galactose, Nieft and Deuel (3) apparently ignored the inverse relationship between the level of fat in the diet and the galactose intake which is now known to affect excretion. Previous workers in this laboratory (1, 2) took cognizance of this factor and in some experiments attempted to equalize the intake per rat. It should be remembered, however, that, while at the beginning of each experiment their rats might have been of equal weight, by the time the urine collections were made, 2 or more weeks later, the rats on the skim milk diets probably weighed less (as was observed in the present studies) and consequently received more galactose per 100 gm. of body weight when the amounts per rat were equalized.

Therefore, it is not surprising that they observed much higher galactosuria in rats on skim milk diets.

#### SUMMARY

1. Studies on the utilization of galactose in rats when this sugar was given in proportion to body weight gave the following results: (a) The excretion of galactose was proportional to the intake of this sugar. (b) A considerably larger fraction was excreted when small amounts were given directly into the stomach in three portions per day than when equal amounts were ingested by the rat in the diet throughout the day. (c) Galactosuria was strikingly lower when equivalent amounts of galactose were fed in the form of lactose.

2. When the influence of fat was compared with that of casein or sucrose on the per cent excretion of galactose, the presence of fat resulted in somewhat lower galactose excretion. This was more noticeable when low levels of galactose were ingested with the diet.

3. Since the influence of fat on the excretion of galactose appears to be small, it is possible that it is exerted largely or entirely by delaying the gastric emptying and by reducing the rate of absorption of galactose.

4. It appears that the major factor influencing the excretion of galactose is the rate at which this sugar reaches the circulation.

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# ISOLATION OF HYALURONIC ACID FROM THE COCK'S COMB\*

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The great size of the cock's comb in contrast to that of the hen or capon is due in large part to the presence of a thick layer of shiny mucinous connective tissue. Champy and Kritch (1) and later Hardesty (2) demonstrated that this swollen connective tissue stains metachromatically with certain basic dyes. Since metachromasia with basic dyes is a well recognized property of mucopolysaccharides (3), we decided to study the chemical nature of the metachromatic comb material.

Hyaluronic acid has been identified in the skin of the pig (4), rabbit (5), and man (6). Since the comb may be considered a modified skin structure, it seemed possible that its metachromatic material might also be hyaluronic acid. Extractions were therefore carried out in order to isolate and identify the comb substance.

The combs were extracted by methods used by Meyer (7) and Meyer and Palmer (8) on umbilical cords and vitreous humor. The dried comb extract represented 0.6 per cent of the initial dried comb weight. Identification of the material extracted was based upon its viscosity, upon the reduction of viscosity and turbidity by the action of hyaluronidase, upon its glucosamine, nitrogen, and acetyl contents, and upon the electrophoretic pattern. The results of these determinations were in close accord with the hyaluronic acid values obtained by others from sources such as umbilical cord and vitreous humor (7-9).

## EXPERIMENTAL

*Methods*—500 gm. of adult cocks' combs (assorted breeds),<sup>1</sup> obtained 4 hours after killing, were ground twice in an electric meat grinder with  $\frac{1}{8}$  inch holes and placed in a liter of acetone in a refrigerator. After 24 hours the acetone was squeezed from the comb material and additional acetone added. This was repeated ten times in 24 hour intervals. After the last extraction the remaining acetone was evaporated in a stream of air. The weight of the dried and defatted combs was 80 gm. This material was extracted ten times successively with a 5 per cent solution of

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<sup>1</sup> Cocks' combs were kindly supplied by Armour and Company, Chicago, and the Oak Valley Farm Products, Inc., New York.

sodium acetate, with 1 liter at 24 hour intervals; each time the viscous fluid was squeezed through several layers of cheese-cloth. Frequent additional macerations in a Waring blender were necessary for complete extraction of the viscous material. The final comb residue was then discarded. 1.5 volumes of ethyl alcohol were added to the aqueous extracts. The precipitates formed were pooled, centrifuged, redissolved in 5 per cent sodium acetate solution, and recentrifuged. Protein was removed from the supernatant solution by shaking it with chloroform four times and then with a chloroform-amyl alcohol (1:4 parts to 1:2 parts) mixture several times until a gel no longer formed. The final solution was dialyzed; sodium acetate crystals were added to make a 5 per cent solution. Following acidification to pH 4.0, the solution was precipitated with ethyl alcohol and the precipitate was desiccated *in vacuo* over calcium chloride. The final dried material weighed 500 mg. and was pure white and fibrous in appearance. It dissolved slowly but completely in water, giving a clear viscous solution.

**Identification**—Viscosity was determined with an Ostwald viscosimeter at a temperature of 37°. With M/15 phosphate buffer of pH 7.0, both as the control flow time and as solute for the unknown, the relative viscosity of the latter at 0.1 gm. per cent concentration was 2.37, and at 0.3 gm. per cent concentration 6.41. Reported values for the viscosity of hyaluronic acid vary from 1.1 to 8.2 at a concentration of 1 gm. per liter (9).

A prompt fall in viscosity resulted from incubating the unknown substrate with hyaluronidase.<sup>2</sup> At the end of 26 minutes, the relative viscosity of the 0.3 gm. per cent solution had fallen from 6.41 to 1.33.

By using a standard turbidimetric assay method<sup>2</sup> with known amounts of hyaluronidase, we compared the relative diminution of turbidity between a known hyaluronate preparation<sup>2</sup> and the comb extract; they were found to be identical.

Glucosamine was determined by the method of Elson and Morgan (10) on material that had been hydrolyzed for 6 hours with 5 N hydrochloric acid at 100° and evaporated to dryness. The glucosamine content of the cock's comb material was 28.4 and 32.7 per cent. (Whereas theory demands 43 per cent, actual analyses of hyaluronic acid range from 20 to 43 per cent and seem to vary with the viscosity of the preparation (9).)

Nitrogen was determined by the micro-Kjeldahl method and values of 3.2 and 3.4 per cent were obtained (theoretical 3.3 per cent).

For the acetyl determinations, the comb material was hydrolyzed for 1½ hours in 2.5 N sulfuric acid at 100° under a reflux condenser. It was then steam-distilled in an apparatus described by Markham (11). The

<sup>2</sup> Bull testis hyaluronidase and potassium hyaluronate were generously supplied by Dr. D. Roy McCullagh of the Schering Corporation, Bloomfield, New Jersey. The turbidimetric method used was that developed at the Schering Corporation.

distillate was titrated against N/70 sodium hydroxide. This method gave acetyl values of 12.2 and 13.2 per cent (theoretical, 10.2 per cent).

A sample of the cock's comb extract was analyzed electrophoretically<sup>a</sup> in a phosphate buffer of pH 8.00, ionic strength 0.10, similar to that used by Blix (12) in studying vitreous humor and synovial fluid hyaluronate. The cock's comb material had a sharp peak with a mobility of 1.16 cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup> 10<sup>-5</sup>. This is in agreement with the value Blix (12) obtained for synovial fluid hyaluronate.

#### DISCUSSION

Hyaluronic acid is present in appreciable quantities in the cock's comb and can be isolated in relatively pure form by the methods outlined here.

The metachromasia observed in the cock's comb with certain basic dyes (toluidine blue, thionine, etc.) appears to be in large part, if not wholly, due to the presence of hyaluronic acid.

In 1934 Berdnikoff and Champy (13) extracted two viscous substances from the cock's comb. One of these extracts contained a reducing sugar resembling glucose. Because of the presence of phosphorus in one extract and appreciable quantities of nitrogen in both, they were led to believe that they had isolated glycoproteins and phosphoproteins. These, they believed, were responsible for the metachromasia observed in the cock's comb. In view of what we now know, in all likelihood they dealt with an impure form of hyaluronic acid.

#### SUMMARY

Hyaluronic acid is present in the cock's comb in appreciable quantity. Aqueous extraction methods were used and its identification was based upon its viscosity, its reaction with hyaluronidase, its glucosamine, nitrogen, and acetyl content, and on its electrophoretic pattern.

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<sup>a</sup> We wish to thank Miss Miriam Reiner for the electrophoresis of the comb material.





# OXIDATIVE AND GLYCOLYTIC METABOLISM OF MINCED DAY-OLD MOUSE BRAIN IN RELATION TO PROPAGATION OF THEILER'S GD VII VIRUS\*

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Repeated failures that have attended attempts to culture various viruses in the absence of living host cells suggest that virus multiplication depends upon metabolic systems in the host tissue. The present study concerns the relationship of oxygen consumption, glucose utilization, and lactic acid production in minced, 1 day-old mouse brain to the *in vitro* propagation of Theiler's GD VII strain of mouse encephalomyelitis virus in this tissue.

## *Materials and Methods*

Brain tissue was removed aseptically from 1 day-old Swiss mice. The brain was minced at room temperature with scissors into small cubes of about 0.5 c.mm., and 50 to 90 mg. of minced tissue were placed in sterile 50 ml. Erlenmeyer flasks, or sterile Warburg vessels containing 2.5 ml. of the appropriate medium. The composition of the medium in the flask experiments was the Simms' solution indicated in Table I, unless otherwise stated. Warburg experiments were mainly carried out with a modified culture medium designated as Solution 23 in Table I. A strain of Theiler's GD VII mouse encephalomyelitis virus which is readily propagated under these conditions (1) was added to the infected series of flasks or Warburg vessels. This virus was obtained from the supernatant fluid of virus-infected, 1 day-old minced mouse brain in Simms' solution. This supernatant was diluted 1000-fold and was added so that the minced tissue suspension had a starting titer of  $10^{-2}$ ; i.e., 100-fold dilution would kill half of the mice injected intracerebrally with 0.3 ml. Control flasks of minced brain suspension received the same amount of supernatant fluid from non-infected tissue. In flask experiments the pH was adjusted to 9 with NaOH and the volume brought to 3 ml. with Simms' solution. Oxygen consumption was measured at 35° by the direct Warburg method

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† With the technical assistance of Alice Guisot and Dorothy Lagerborg.

with potassium hydroxide in the center cup and an atmosphere of air. The flask experiments were carried out at 35° in closed vessels in an atmosphere of air without agitation. All the flasks were tested and found sterile at the end of the incubation period. Tissue was removed by centrifugation for 10 minutes at a centrifugal force of 900 times gravity in a horizontal head. The supernatant fluid was used for determination of virus titer and glucose and lactic acid content. The virus titer was determined by intracerebral injection of 0.03 ml. of the diluted supernatant into mice, the titer being expressed as the highest dilution which was lethal to at least half of a group of six to eight mice.

1 ml. of the supernatant was added to 9 ml. of freshly prepared tungstic acid reagent (5 ml. of 0.66 N  $\text{H}_2\text{SO}_4$  plus 5 ml. of 10 per cent  $\text{NaWO}_4$  and diluted to 50 ml.). The precipitated proteins were centrifuged off, and

TABLE I  
*Composition of Culture Media*

Constituent	Simms' solution X7	Solution 23
	<i>gm. per l.</i>	<i>gm. per l.</i>
NaCl.....	8.0	8.0
KCl.....	0.2	0.2
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ .....	0.147	0.147
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ .....	0.203	0.203
$\text{NaHCO}_3$ .....	1.01	0.505
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ..	0.213	
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ...		0.05
Glucose.....	1.0	2.0
Phenol red.....	0.01	0.01

glucose and lactic acid determinations carried out on the supernatant by the methods of Folin and Malmros (2) and of Barker and Summerson (3) respectively. Differences in the medium or in the conditions of incubation are indicated in the data for specific experiments. All metabolic values are expressed as quantities per 100 mg. of fresh tissue.

### *Results*

The oxidative and glycolytic metabolism of sterile minced mouse brain tissue as a function of time and the influence of virus upon this metabolism have been studied in Warburg and in flask experiments. Fig. 1 shows a typical experiment in which the oxygen consumption of 1 day mouse brain was studied in the presence and absence of virus for a period of 70 hours. It is seen that the rate of metabolism falls rapidly with time and reaches low values by the end of 24 hours. The  $\text{Q}_{\text{O}_2}$  of brain minces inoculated

with virus was not significantly different from the uninoculated tissue except after 48 hours when the infected tissues occasionally showed lower rates than the controls.

In experiments of the type shown in Fig. 1, the disappearance of glucose and the production of lactic acid and virus were determined at the end of the Warburg experiment. The results of typical experiments are shown in Table II. The relationship of metabolism to incubation time and virus propagation has also been studied with the flask technique, the results

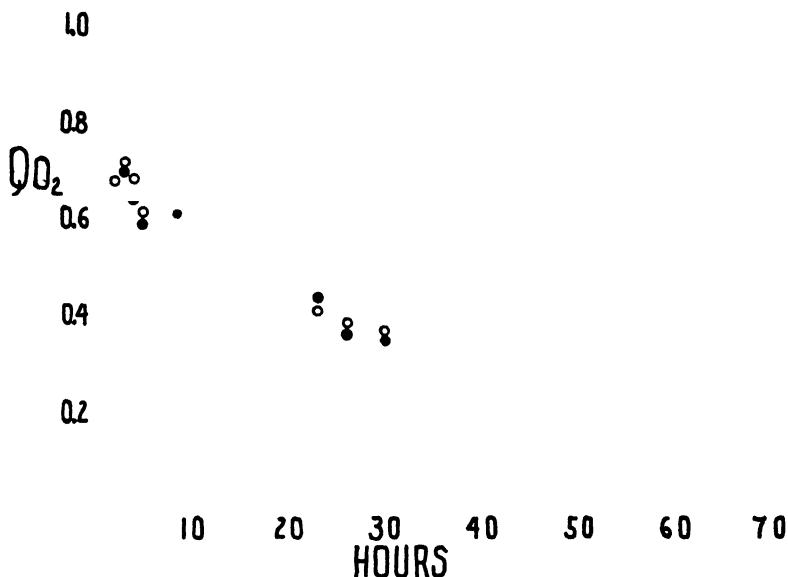


FIG. 1. The oxygen consumption of minced mouse brain. 50 to 80 mg. of sterile minced mouse brain were placed in 3 ml. of Solution 23 and the oxygen consumption followed manometrically at 35°. O, 1 day-old mouse brain; ●, 1 day-old mouse brain infected with Theiler's GD VII virus. The values on the ordinate are expressed as microliters of O<sub>2</sub> used per hour per mg. of wet tissue.

being summarized in Fig. 2. No differences were observed between infected and control tissue. These data indicate that the lactic acid produced by 1 day-old mouse brain under the conditions of these experiments is usually slightly in excess of the glucose which disappears. When the oxygen consumption is expressed in terms of the amount of glucose oxidized, the discrepancy between the glucose used and that calculated from the end-products is increased. This is, of course, interpreted as an indication that endogenous substrates are available both for glycolysis and for respiration in these long term experiments.

TABLE II

*Utilization of Oxygen and Glucose and Production of Lactic Acid and Virus by 1 Day-Old Mouse Brain in Warburg Experiments*

Tissue and treatment	Incubation	Total oxygen used	Glucose equivalent oxygen	Total lactic acid produced	Total glucose disappeared	Final pH	Virus titer*
	hrs.	c.mm. per 100 mg.	mg. per 100 mg.	mg. per 100 mg.	mg. per 100 mg.		
1 day-old brain + virus	21	850	1.14	1.93	1.76	8.6	$10^{-4}$
1 " " no virus..	21	830	1.11	1.85	1.80	8.6	
1 " " + virus....	70	1950	2.61	3.90	3.45	7.5	$10^{-5}$
1 " " no virus .	70	1800	2.41	3.28	3.18	7.9	

Approximately 50 mg. of 1 day-old mouse brain in 3 ml. of Solution 23 with and without virus. The experiments were carried out at 35° in an atmosphere of air.

\* Highest dilution that killed at least half of a group of six to eight mice after intracerebral injection of each with 0.03 ml.

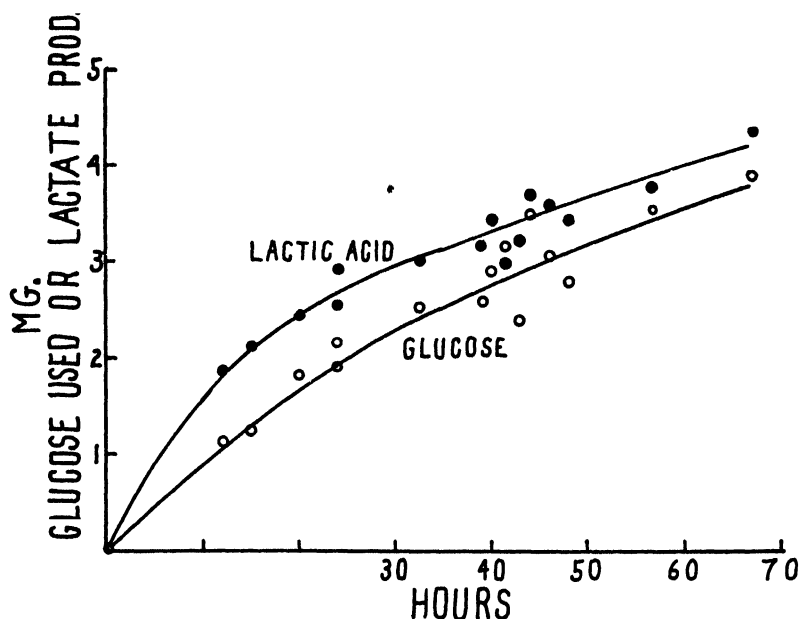


FIG. 2. The effect of Theiler's GD VII virus on glucose utilization and lactic acid production by 50 to 80 mg. of 1 day minced mouse brain incubated in Simms' solution at an initial pH of 9 at 35° in flask experiments.

It is to be noted from Figs. 1 and 2 that the oxidative and glycolytic metabolism of mouse brain minces falls off considerably after 12 to 24 hours. In spite of this diminished metabolism, it has previously been ob-

served (1) that virus added to minced brain tissue, preincubated at 35° from 2 to 10 days, multiplies almost as well as when the virus is added to the freshly prepared brain tissue. This is also shown in Table III.

TABLE III

*Propagation of Theiler's GD VII Virus in Minced Mouse Brain Preincubated for Various Times*

Incubation with virus was accomplished in 3 days.

Tissue		Final virus titer*
1	day-old mouse brain not preincubated	10 <sup>-6</sup>
1	" " " preincubated† 2 days	10 <sup>-6</sup>
1	" " " " 5 "	10 <sup>-4</sup>
1	" " " " 10 "	10 <sup>-4</sup> -10 <sup>-4</sup>
1	" " " " 20 "	10 <sup>-2</sup>

\* The initial titer of virus is 10<sup>-2</sup>.

† Preincubated at 35° in an atmosphere of air without agitation before the addition of the virus.

#### DISCUSSION

It is evident from this work that the oxidative or glycolytic metabolism of minced 1 day-old mouse brain tissue is not affected significantly by the propagation of Theiler's GD VII virus during the time when the virus is growing at a maximal rate.

It is of interest that virus propagation occurs in spite of the decrease in rates of oxygen consumption, glucose utilization, or lactic acid production of cultures of 1 day-old mouse brains preincubated for as long as 10 days before addition of virus. It would appear that the rate of carbohydrate metabolism of the host tissue is not of paramount importance in determining the growth of the virus.

Racker and Kabat (4) and Nickle and Kabat (5) have reported that the anaerobic glycolysis rate of mouse brain taken from mice showing paralysis following intracerebral injection with neurotropic viruses was lower than the rates for brains of uninoculated controls. Wood, Rusoff, and Reiner (6), however, were unable to verify these findings. The phosphorylation of glucose appeared to be inhibited in homogenates of mouse brain infected with Lansing or Theiler's F. A. viruses (Racker and Krinsky (7)). Experiments with brain tissue taken from infected adult mice are not comparable with those reported here. However, even in our experiments, where the proportion of cells affected by the virus may be expected to be maximal, the effect of virus propagation on oxygen consumption, glucose utilization, or lactic acid production has not been demonstrable. The failure of Theiler's virus propagation to influence oxidation or glycolysis rates of 1 day-

old mouse brain minces is of interest in view of the marked effects of this virus on the turnover of the phospholipide and protein-bound phosphate fractions of mouse brain and on the assimilation of radioactive glucose fragments reported elsewhere (8, 9).

#### SUMMARY

The oxygen consumption, glucose utilization, lactic acid production, and propagation of Theiler's GD VII mouse encephalomyelitis virus in minced 1 day-old mouse brain have been studied. The propagation of the virus had no significant influence on the oxidative or glycolytic metabolism of the host tissue. The rate of oxidative or glycolytic metabolism of the host tissue was not of prime importance in affecting the propagation of the virus.

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# THE EFFECTS OF THEILER'S GD VII VIRUS ON $P^{32}$ UPTAKE BY MINCED ONE DAY-OLD MOUSE BRAIN\*

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Previous studies in this laboratory have shown that the propagation of Theiler's GD VII strain of mouse encephalomyelitis virus in minced 1 day-old mouse brain had no effect on oxygen consumption, glucose utilization, or lactic acid production of the host tissue (1). It was also concluded that a high rate of glycolysis or respiration in the host tissue was not of primary importance in determining growth of the virus, since brain tissue preincubated for 10 days prior to the introduction of virus had low rates of glycolysis and respiration, but would still support virus growth. It seemed possible that synthetic reactions might be directly influenced by the virus. We have therefore undertaken a study of the rate of incorporation of  $P^{32}$ -labeled inorganic phosphate into various phosphate fractions of 1 day-old minced mouse brain and the effects of the propagation of Theiler's GD VII virus on these processes.

## *Methods and Materials*

*Preparation of Cultures and Incubation Conditions*—40 to 60 mg. of weighed, minced brain tissue aseptically removed from day-old mice were placed in a 50 ml. Erlenmeyer flask containing 2.5 ml. of Simms' solution (1) and 1  $\mu$ c. of  $P^{32}$  as inorganic phosphate. Theiler's GD VII virus grown in minced mouse brain was added, so that 0.03 ml. of the supernatant fluid diluted 100-fold was lethal for half the mice injected intracerebrally. Increase in the virus inoculum by 10-fold or a reduction by 100-fold did not alter the results obtained. Control flasks received the same amount of material from non-infected brain minces. The reaction of the flasks was adjusted to pH 9 by the addition of dilute NaOH. The final volume of each flask was 3.0 ml. The flasks were closed with rubber

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stoppers and incubated without shaking at 35° for varying periods of time. At the termination of the incubation period all flasks were tested for sterility.

*Determination of Virus Titer*—After the incubation period 0.5 ml. of the supernatant fluid was aseptically removed from the flasks without agitation and its virus titer determined by injecting 0.03 ml. of serial 10-fold dilutions intracerebrally into groups of six mice 5 to 7 weeks old. The end-point employed was the dilution at which at least half of the mice died in 3 to 15 days.

*Fractionation of Tissue*—Two fractionation procedures differing only in the extent of fractionation of the total protein-bound phosphate fractions were used.

The contents of two incubated flasks were combined and transferred quantitatively with water to graduated centrifuge tubes. Trichloroacetic acid (TCA) was added to a final concentration of 5 per cent. The tubes stood, with occasional shaking, for 30 minutes at room temperature and were then centrifuged. This yielded two main fractions, the total acid-soluble (TAS) and the acid-insoluble residue (R). The residue was washed three times with 15 ml. of 5 per cent TCA for 15 minutes each time and the washes discarded. After a sample of the TAS was removed to determine total phosphorus in that fraction, the fraction was separated into the inorganic orthophosphate (IP) and organic acid-soluble (OAS) fractions by the method of Delory (2). Preliminary studies had shown that, while the Delory procedure coprecipitates some ester-bound phosphorus, a more complete precipitation of inorganic orthophosphate was obtained than with the alkaline magnesia method. A minor correction was applied to IP and OAS after digestion of the Delory precipitate.

The lipide-bound phosphate (LP) was extracted from R by shaking 2 hours with 5 ml. of 3:1 ethanol-ether and refluxing at 70° for 30 minutes with 5 ml. of 1:1 chloroform-methanol. The remaining residue, the "total protein-bound" phosphate fraction (TPP), was washed twice with 5 ml. of ether and dried *in vacuo*.

In a second series of experiments the TPP fraction was further fractionated by combining certain portions of the methods of Schmidt and Thannhauser (3) and of Schneider (4). The TPP fraction was treated for 14 hours at 35° with 1 N NaOH (phosphate-free). After a sample was removed for the determination of total protein-bound phosphorus, the solution was acidified with 0.1 volume each of concentrated HCl and 50 per cent TCA to precipitate desoxyribonucleic acid (DNA) and residual protein (RP). The acid supernatant contained ribonucleic acid (RNA) and inorganic phosphate (PIP), presumably liberated from phospho-

protein during treatment. The PIP fraction was extracted from the RP by two 15 minute treatments with 5 per cent TCA at 90°. Repetition of the entire fractionation, beginning with the initial treatment of the RP fraction with 1 N NaOH, yielded no additional amounts of RNA or DNA phosphorus. In experiments designed to test the validity of the RNA and DNA phosphorus fractionations, pentose and desoxypentose determinations with the orcinol-HCl and diphenylamine reactions (5, 6) were carried out simultaneously with phosphorus determinations by the fractionation procedure on 1 gm. quantities of 1 day-old mouse brain. Table I shows the excellent agreement obtained with these methods. In the ensuing experiments, phosphorus determinations alone have been used as a quantitative estimate of the RNA and DNA present in each experiment. This procedure permits the independent determination of  $P^{31}$  and  $P^{32}$  in each fraction.

TABLE I

*Comparison of Nucleic Acid Phosphorus Values Obtained by Phosphorus Estimations and As Calculated from Pentose and Desoxypentose Determinations*

The values are given in micrograms of P per 100 mg. of fresh tissue.

RNA		DNA	
By phosphorus	By pentose	By phosphorus	By desoxypentose
16.5	16.0	13.0	13.7
18.4	17.5	14.2	13.8
17.1	16.9	13.6	13.3

*$P^{31}$  and  $P^{32}$  Analyses*—The chemical analysis for  $P^{31}$  was carried out by a modification of the method of Fiske and Subbarow (7) in which ascorbate was used as a reducing agent and a heating period was employed for color development and stabilization.

Radioactive samples were prepared in 0.25 ounce tin ointment dishes. The samples were dried under an infra-red lamp and counted with a mica window tube on the same day, thereby negating decay or counter-corrections. Counts were always many times the background and a minimum of 3000 counts was always taken.

*Expression of Results*— $P^{31}$  results are expressed as micrograms of phosphorus per 100 mg. of fresh tissue weight. Protein nitrogen determinations by the method of Sobel, Mayer, and Gottfried (8) yielded a good correlation with fresh tissue weight as an index of the amount of active tissue present.

$P^{32}$  results are expressed in terms of relative specific activity (RSA),

i.e. the specific activity (counts per minute per microgram of  $P^{31}$ ) of each fraction as per cent of the specific activity of the IP fraction.<sup>1</sup>

#### EXPERIMENTAL

*Effects of Incubation Time and Virus Propagation on  $P^{32}$  Uptake*—Table II shows the data for the average and the range of uptake of  $P^{32}$  into the LP, TPP, and OAS fractions as a function of incubation time and as influenced by virus propagation. In both infected and non-infected brain, the relative specific activities of these fractions increased initially with incubation, reached a maximum in 24 to 48 hours, and then decreased with further incubation.

Virus propagation markedly stimulated the uptake of  $P^{32}$  into the LP and TPP fractions in the first 6 to 24 hours of incubation, whereas there was no significant effect on the uptake into the OAS fraction (Table II). These differences are well outside any experimental errors. It is concluded that during the period of virus propagation the turnover of phospholipides and protein-bound phosphorus is greater than for uninfected controls. It is of interest that this first 24 hour interval is the period of maximal virus production, as can be seen from Table III, which presents data showing the relation of virus titer to incubation time. Although the effect of the virus on  $P^{32}$  uptake is apparent in the 6 and 12 hour intervals, no liberation of virus into the supernatant fluid is apparent in this interval (Table III). The explanation for this phenomenon is as yet not apparent.

At 36 and 48 hours the difference between infected and non-infected tissues became less significant. After 72 and 120 hours of incubation the relation was reversed with the LP and TPP fractions having relative specific activities higher in the non-infected than in the infected tissues. This reversal appears to be due to a more rapid fall in relative specific activity in the infected tissues. No difference in the relative specific activities of the OAS fraction between the infected and non-infected tissues was seen at any incubation time.

Table IV shows the variations in phosphorus content for the experiments reported in Table II. No significant differences were found in these three fractions between infected and non-infected tissues.

A certain amount of contamination of the phospholipide and total protein-bound phosphate fraction was observed when labeled inorganic phosphate was added to new-born mouse brain mince and the tissue

<sup>1</sup> In the experiments included in this work 95 to 98 per cent of the added radioactivity was recovered in the fractions and washings analyzed. 90 per cent of this was in the inorganic phosphate fraction in the medium, since this contained 8 times the amount of the combined OAS, LP, and TP fractions.

**Effects of Virus Propagation on Incorporation of  $P^{32}$  into Phospholipide, Total Protein-Bound, and Organic Acid-Soluble Phosphate Fractions of Day-Old Mouse Brain in Tissue Culture**

Incubation hrs.	No. of experiments	Relative specific activities					
		LP		TPP		OAS	
		No virus	Virus	No virus	Virus	No virus	Virus
6	6 Average Range	1.25 ± 0.08* (0.9 - 1.4)	2.12 ± 0.12 (1.7 - 2.4) $P = <0.001$ †	0.78 ± 0.04 (0.7 - 0.9)	1.17 ± 0.04 (1.1 - 1.3) $P = <0.001$	6.29 ± 0.37 (4.5 - 7.9)	7.51 ± 0.20 (6.7 - 8.5) $P = 0.018$
12	19 Average Range	2.05 ± 0.04 (1.7 - 2.3)	2.95 ± 0.04 (2.6 - 3.4) $P = <0.001$	1.47 ± 0.05 (1.0 - 1.8)	2.37 ± 0.04 (2.1 - 2.6) $P = <0.001$	17.51 ± 0.84 (11.4 - 26.9)	18.70 ± 0.35 (11.5 - 24.1) $P = 0.204$
24	18 Average Range	2.91 ± 0.04 (2.8 - 3.1)	4.15 ± 0.05 (4.1 - 4.5) $P = <0.001$	2.38 ± 0.03 (2.3 - 3.3)	3.54 ± 0.07 (3.3 - 4.7) $P = <0.001$	53.53 ± 2.60 (51.7 - 60.6)	51.69 ± 1.40 (49.7 - 54.2) $P = 0.271$
36	9 Average Range	6.65 ± 0.15 (6.0 - 7.2)	5.57 ± 0.15 (4.6 - 6.1) $P = <0.001$	5.56 ± 0.21 (5.1 - 6.4)	5.38 ± 0.11 (5.0 - 5.9) $P = >0.553$	11.34 ± 0.37 (10.2 - 13.1)	12.97 ± 0.61 (10.8 - 16.2) $P = 0.054$
48	11 Average Range	10.21 ± 0.54 (7.8 - 12.3)	8.01 ± 0.12 (7.3 - 8.5) $P = 0.009$	4.28 ± 0.11 (3.9 - 5.0)	4.53 ± 0.12 (3.8 - 5.1) $P = 0.125$	12.52 ± 0.86 (9.3 - 18.6)	12.20 ± 0.77 (8.8 - 16.8) $P = >0.555$
72	11 Average Range	5.90 ± 0.49 (4.0 - 7.8)	3.15 ± 0.15 (2.4 - 3.8) $P = <0.001$	3.88 ± 0.32 (2.8 - 5.3)	1.85 ± 0.15 (1.1 - 2.6) $P = <0.001$	3.96 ± 0.16 (3.1 - 4.7)	3.81 ± 0.14 (2.6 - 4.7) $P = 0.493$
120	11 Average Range	4.94 ± 0.11 (4.4 - 5.4)	2.55 ± 0.11 (1.9 - 3.2) $P = <0.001$	1.90 ± 0.26 (1.0 - 3.0)	1.42 ± 0.09 (1.0 - 1.9) $P = 0.009$	2.89 ± 0.18 (2.4 - 3.8)	2.89 ± 0.12 (2.3 - 3.3)

\* Standard error of the mean calculated from s.e.m. =  $\sqrt{(\bar{x}^2/(n-1))/n}$ .

† Calculated after Fisher for small sample statistics.

TABLE III  
*Relation of Virus Titer to Incubation Time*

Incubation	Virus titer*
<i>hrs.</i>	
0	10 <sup>-2</sup>
6	10 <sup>-2</sup>
12	10 <sup>-2</sup>
18	10 <sup>-4</sup>
24	10 <sup>-5</sup> -10 <sup>-6</sup>
36	10 <sup>-5</sup> -10 <sup>-6</sup>
48	10 <sup>-5</sup> -10 <sup>-6</sup>
72	10 <sup>-5</sup> -10 <sup>-6</sup>
120	10 <sup>-5</sup> -10 <sup>-6</sup>

\* Highest dilution that killed at least half of a group of six to eight mice after intracerebral injection of each with 0.03 ml.

TABLE IV  
*Total Phosphate Content of Phospholipide, Total Protein-Bound, and Organic Acid-Soluble Fractions at Various Incubation Times*

The values are given as micrograms of P per 100 mg. of tissue.

In-cubation	No. of ex- peri- ments	LP		TPP		OAS	
		No virus	Virus	No virus	Virus	No virus	Virus
<i>hrs.</i>							
0	3	61.0 ± 1.0*		81.0 ± 1.0		88.0 ± 1.2	
6	6	43.0 ± 2.6	52.0 ± 7.2 <i>P</i> = 0.258†	55.0 ± 1.7	46.0 ± 3.4 <i>P</i> = 0.037	44.0 ± 1.7	42.0 ± 1.0 <i>P</i> = 0.341
12	19	46.0 ± 1.8	44.0 ± 2.1 <i>P</i> = 0.194	42.0 ± 1.9	40.0 ± 1.3 <i>P</i> = 0.368	34.7 ± 1.2	31.0 ± 1.7 <i>P</i> = 0.218
24	16	50.0 ± 1.1	48.0 ± 1.5 <i>P</i> = 0.280	45.0 ± 1.2	43.0 ± 1.6 <i>P</i> = 0.325	31.0 ± 1.0	25.0 ± 1.1 <i>P</i> = 0.055
36	7	60.0 ± 3.6	58.0 ± 2.3 <i>P</i> = 0.570	44.0 ± 0.7	46.0 ± 2.4 <i>P</i> = 0.454	22.0 ± 0.7	17.0 ± 0.5 <i>P</i> = 0.097
48	11	54.0 ± 2.2	57.0 ± 2.3 <i>P</i> = 0.378	43.6 ± 2.1	44.1 ± 2.3 <i>P</i> = 0.555	18.0 ± 0.9	15.0 ± 1.0 <i>P</i> = 0.244
72	7	46.0 ± 1.6	44.0 ± 2.1 <i>P</i> = 0.570	41.1 ± 1.0	39.6 ± 1.2 <i>P</i> = 0.454	11.1 ± 0.9	10.0 ± 1.2 <i>P</i> = 0.560
120	7	36.0 ± 3.3	31.0 ± 4.7 <i>P</i> = 0.378	34.0 ± 1.5	29.0 ± 4.1 <i>P</i> = 0.337	7.2 ± 1.2	6.3 ± 0.9 <i>P</i> = 0.439

\* Standard error of the mean calculated from  $s.e.m. = \sqrt{(d^2/(n-1))/n}$ .

† Calculated after Fisher for statistics on small samples.

treated immediately with TCA. The relative specific activities of the acid-insoluble residue phosphate (R) were  $0.20 \pm 0.02$  in eight experi-

ments. The amount of  $P^{32}$  adsorption appeared to be consistent and insufficient to impair the validity of the conclusions drawn. The adsorption of organic acid-soluble phosphate has also been considered. The OAS filtrate was taken from tissue incubated for 24 hours in  $P^{32}$ . This filtrate was neutralized, added to both fresh virus-infected brain tissue minces and virus-infected minces incubated 24 hours, and fractionated immediately. The relative specific activity of the acid-insoluble residue was insignificant, being less than 0.01 for both types of experiments. This experiment would indicate that absorption of organic acid-soluble phosphate is not a factor which would interfere with the conclusions reached.

*Participation of Various Protein-Bound Fractions in Increased Activity Due to Virus Propagation*—Table V shows the relative specific activities and the distribution of phosphorus in the various protein-bound phosphate fractions of brain tissue after 12 and 24 hours of incubation. The relative specific activities of the RNA at both 12 and 24 hours and of the PIP at 12 hours were significantly higher in the virus-infected tissue than in the non-infected tissue. No significant differences were found in the DNA or RP fractions. In the infected preparation the amount of RNA phosphorus at both 12 and 24 hours is significantly above and the amount of DNA phosphorus at 24 hours significantly below the uninfected controls.

It is seen from Table V that the specific activity of the RNA fraction accounts for the major portion of the increased  $P^{32}$  uptake by the virus-infected tissue, since this fraction is present in highest amount and has a high specific activity. In 12 hours over half the effect of virus is on the RNA uptake of  $P^{32}$ ; at 24 hours 83 per cent of the effect is on the turnover of the RNA fraction.

The highest relative specific activity of any protein-bound phosphate fraction was found in the PIP fraction. In separate experiments in which  $P^{32}O_4$  was added to 1 day-old mouse brain and fractionated immediately by the above procedure, it was found that radioactive phosphate was found only in the PIP fraction and not in DNA, RNA, or RP. Thus it is concluded that inorganic phosphate is carried by absorption into this fraction and is freed by the NaOH and TCA treatments. The PIP fraction cannot be solely due to adsorbed inorganic phosphate, since its relative specific activity would then be 100.

*Effect of Preincubation on  $P^{32}$  Uptake and on Its Stimulation by Virus*—It has previously been observed (1, 9) that 1 day-old mouse brain incubated at  $35^\circ$  for periods up to 10 days was capable of supporting growth of Theiler's virus. It was of interest to determine whether in such preincubated tissue the virus would exert its stimulating action on phos-

pholipide and total protein-bound phosphate metabolism. Table VI gives the data from 24 hour experiments in which  $P^{32}$ -labeled phosphate

TABLE V

*Effects of Virus Propagation on Uptake of  $P^{32}$  and Distribution of Phosphorus in the Various Protein-Bound Fractions*

		Relative specific activity		P per 100 mg. tissue, $\gamma$	
		12 hrs.	24 hrs.	12 hrs.	24 hrs.
TPP	No virus	1.20 $\pm$ 0.01* (1.1 - 1.4)† $P = <0.001$ ‡	2.32 $\pm$ 0.16 (2.1 - 2.7) $P = <0.001$	36.3 $\pm$ 0.6 (35 - 39) $P = 0.04$	40.2 $\pm$ 0.9 (39 - 42) $P = 0.12$
	Virus	2.15 $\pm$ 0.01 (2.0 - 2.3)	3.55 $\pm$ 0.17 (3.4 - 3.9)	38.6 $\pm$ 0.8 (38 - 43)	42.8 $\pm$ 0.3 (39 - 43)
RNA	No virus	1.16 $\pm$ 0.04 (1.1 - 1.3) $P = <0.001$	2.38 $\pm$ 0.04 (2.3 - 2.6) $P = <0.001$	15.3 $\pm$ 0.5 (14 - 17) $P = 0.001$	15.5 $\pm$ 0.5 (13 - 17) $P = 0.001$
	Virus	2.09 $\pm$ 0.06 (1.9 - 2.3)	3.90 $\pm$ 0.07 (3.9 - 4.1)	18.8 $\pm$ 0.6 (17 - 21)	21.4 $\pm$ 1.2 (18 - 26)
DNA	No virus	0.54 $\pm$ 0.01 (0.5 - 0.6) $P = 0.165$	1.16 $\pm$ 0.04 (1.0 - 1.2) $P = >0.562$	8.3 $\pm$ 0.3 (7.0 - 9.1) $P = 0.044$	13.0 $\pm$ 0.3 (12.0 - 14.0) $P = 0.001$
	Virus	0.61 $\pm$ 0.04 (0.5 - 0.8)	1.18 $\pm$ 0.04 (1.0 - 1.3)	9.7 $\pm$ 0.5 (8.9 - 11.1)	9.6 $\pm$ 0.5 (7.9 - 11.0)
RP	No virus	0.83 $\pm$ 0.04 (0.7 - 1.0) $P = 0.562$	0.84 $\pm$ 0.03 (0.8 - 1.0) $P = >0.562$	4.4 $\pm$ 0.3 (3.1 - 5.1) $P = >0.562$	5.5 $\pm$ 0.5 (4.9 - 7.1) $P = >0.562$
	Virus	0.84 $\pm$ 0.03 (0.8 - 1.0)	0.89 $\pm$ 0.02 (0.8 - 1.0)	4.8 $\pm$ 0.3 (4.0 - 6.0)	4.5 $\pm$ 0.5 (3.0 - 5.9)
PIP	No virus	1.67 $\pm$ 0.19 (1.1 - 2.3) $P = 0.001$	4.57 $\pm$ 0.25 (3.7 - 6.2) $P = >0.562$	7.6 $\pm$ 0.5 (7.1 - 9.0) $P = >0.562$	6.8 $\pm$ 0.6 (5.0 - 8.1) $P = >0.562$
	Virus	3.62 $\pm$ 0.39 (2.8 - 5.1)	4.94 $\pm$ 0.4 (4.0 - 6.7)	8.0 $\pm$ 0.1 (7.3 - 9.0)	7.1 $\pm$ 0.6 (5.8 - 9.6)

The results are averages of six experiments of each group.

\* Standard error of the mean from  $s.e.m. = \sqrt{(d^2/(n-1))/n}$ .

† Range of values for each group.

‡ Calculated after Fisher for statistics on small samples.

and virus were added to 1 day-old brain minces preincubated for 0, 5, 10, or 15 days at 35°. The 24 hour uptake of  $P^{32}$  into the LP and TPP

fractions progressively declined as the preincubation period was prolonged. The virus stimulated the  $P^{32}$  turnover of both fractions at 0, 5, and 10 but not at 15 days of preincubation. This virus effect appears to correspond roughly to the ability of the virus to grow in these preparations.

TABLE VI

$P^{32}$  Uptake in Day-Old Mouse Brain Cultures Preincubated Varying Period of Time Prior to Introduction of Virus and  $P^{32}$

Preincubation*		Relative specific activities				
		LP		TPP		Virus titer†
		No virus	Virus	No virus	Virus	
days						
0	Average	2.91	4.15	2.38	3.54	10 <sup>-5</sup>
5		2.09	3.40	1.02	1.65	10 <sup>-4</sup>
		2.20	3.28	1.00	1.53	
		2.00	3.19	0.88	1.70	
	Average	2.10	3.29	0.97	1.64	
10		1.82	2.35	0.83	1.43	10 <sup>-4</sup>
		1.67	2.71	0.74	1.34	
		1.73	2.46	0.70	1.29	
	Average	1.74	2.51	0.76	1.35	
15		1.68	1.56	0.83	0.63	10 <sup>-3</sup>
		1.43	1.39	0.74	0.76	
		1.38	1.42	0.59	0.68	
	Average	1.49	1.46	0.72	0.69	

\*  $P^{32}$  and virus are added at the end of this period and preparations are incubated for 24 hours longer.

† Highest dilution that killed at least half of a group of six to eight mice after intracerebral injection of each with 0.03 ml.

## DISCUSSION

It has been shown that during the period of maximal virus propagation the increased uptake of  $P^{32}$  into the TPP fraction is due almost entirely to the increased turnover of RNA phosphorus, and that the slower turnover of DNA phosphorus is not affected by virus propagation. Furthermore, the amount of RNA was increased and that of DNA decreased



in the infected minced tissue, as compared to non-infected controls. These observations may be of significance in regard to the type of nucleic acid in Theiler's virus. In somewhat parallel experiments Cohen (10) has studied the phosphate metabolism of *Escherichia coli* infected with  $T_2$  bacteriophage. 99 per cent of the total phage phosphorus could be accounted for by its DNA content. The metabolism of RNA, which is synthesized in 3 times the quantity of DNA by normal *E. coli*, was found to be essentially inert in the phage-infected *E. coli*, indicating that the phage had redirected the nucleoprotein metabolism of the infected cells. Hyden (11), using microspectrographical and cytochemical methods, has shown that viruses in general cause the production of nucleotides in the host tissue of the specific type contained in the infecting virus. The present finding of both an increased turnover and an increased net amount of RNA may indicate that Theiler's GD VII virus is an RNA type virus.

It would be premature to hazard the suggestion that the increased turnover and amount of RNA in the virus-infected tissue represent the contribution of the virus itself. It has not been possible to isolate a pure virus preparation from these minces or to determine its composition and its  $P^{32}$  content.

At the present time no information is available as to the significance of the increased turnover of the phospholipide fraction brought about by the virus. Studies on the nature of this fraction are contemplated.

A puzzling aspect of the present experiments is the fall in relative specific activities of the LP and TPP fractions with prolonged incubation times. A possible explanation for this effect might involve the selective breakdown of newly formed phospholipide and nucleoprotein during longer incubation periods.

#### SUMMARY

The uptake of  $P^{32}$ -labeled inorganic orthophosphate and the distribution of phosphorus in the various trichloroacetic acid-insoluble phosphate fractions of minced 1 day-old mouse brain and the effect of Theiler's GD VII virus propagation have been studied. During the period of maximal rate of virus propagation the uptake of  $P^{32}$  was markedly stimulated in the phospholipide and "total protein-bound" phosphate fractions. Increased turnover in the "total protein-bound" fraction associated with virus propagation is due primarily to the increased turnover of ribonucleic acid phosphorus. Desoxyribonucleic acid phosphorus turnover was not affected. The virus-infected tissue as compared to the non-infected tissue had an increased ribonucleic acid-phosphorus content and a decreased desoxyribonucleic acid-phosphorus content.

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# THE EFFECTS OF THEILER'S GD VII VIRUS ON THE INCORPORATION OF RADIOACTIVE CARBON FROM GLUCOSE INTO MINCED ONE DAY-OLD MOUSE BRAIN\*

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Previous studies in this laboratory have shown that the propagation of Theiler's GD VII strain of mouse encephalomyelitis virus in minced, 1 day-old mouse brain had no effect on the rates of oxidative or glycolytic metabolism of these minces (1). In spite of the failure to influence respiration and glycolysis, it was found that the virus markedly stimulated the incorporation of  $P^{32}O_4$  into the phospholipides and ribose nucleic acids of the host tissue (2). This observation has led us to investigate the effect of virus propagation on other synthetic processes of the infected brain tissue. This paper describes the effects of Theiler's GD VII strain of mouse encephalomyelitis virus on the incorporation of radioactive carbon from labeled glucose into the tissue components of minced, 1 day-old mouse brain.

## Methods and Materials

*Preparation and Incubation of Tissues*—Control and virus-infected minced, 1 day-old mouse brain was prepared as previously described (1, 2) in sterile 50 ml. Erlenmeyer flasks containing Simms' solution (1) and 3 mg. of  $C^{14}$ -containing glucose prepared photosynthetically with sweet potato leaves by a modification of the method of Putman *et al.* (3).<sup>1</sup> The pH of the minced tissues was adjusted to 9 with dilute NaOH and the volume made up to 3 ml. The flasks were closed with rubber stoppers and incubated at 35° for 24 hours. After removal of the free  $CO_2$  by aeration into dilute NaOH, all flasks were tested for sterility and

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† With the technical assistance of Dorothy Lagerborg.

<sup>1</sup> The glucose used in this work had calculated specific activities of 0.25 to 0.4  $\mu$ c. per mg. With the tube and geometry of our system, such material gave 5.5 to 8.8  $\times 10^4$  counts per minute per mg. corrected to a sample thickness of 0.5 mg. per sq. cm.

virus titer was determined on the supernatant as previously reported (1, 2). The virus titer at zero time was  $10^{-2}$  and at 24 hours was  $10^{-5}$ , corresponding to a 1000-fold increase during the 24 hour incubation period.

*Fractionation of Tissue*—Trichloroacetic acid (TCA) was added to the incubated flasks to a final concentration of 5 per cent and the evolved  $\text{CO}_2$  was aerated into the previous NaOH solution for 30 minutes. The trichloroacetic acid-soluble fraction (AS) was separated from the acid-insoluble residue (R) by centrifugation. The residue was washed three times for 15 minutes each with 10 ml. of 5 per cent TCA and the washes discarded. The lipid fraction (L) was extracted from the residue (R) by shaking for 2 hours with 5 ml. of 3:1 ethanol-ether mixture and refluxing twice for 20 minutes at  $70^\circ$  with 5 ml. portions of 1:1 chloroform-methanol. The remaining "protein fraction" (P) was washed twice with 5 ml. portions of ether and dried *in vacuo*. The P fraction was then solubilized by treatment with 1 N NaOH for 14 hours at  $35^\circ$ .

*C<sup>14</sup> Analysis*—Duplicate aliquots of each fraction were dried directly on aluminum disks over an area of 15.9 sq. cm. and counted with a thin mica window tube. All samples were less than 1 mg. per sq. cm. in thickness and were corrected to 0.5 mg. per sq. cm. All samples contained counts at least 3 times that of the background and a minimum of 2000 counts was taken.

*Expression of Results*—The results are expressed as the recovery in each fraction ( $\text{CO}_2$ , AS, L, and P) in per cent of the radioactivity initially added as glucose.

#### EXPERIMENTAL

*Incorporation of Radioactive Carbon from Labeled Glucose*—Table I gives data from eighteen different experiments for the incorporation of radio-carbon from glucose into the  $\text{CO}_2$ , acid-soluble, lipid, and "protein" fractions of minced, 1 day-old mouse brain incubated for 24 hours with 3 mg. of labeled glucose. Some 91 per cent of the original  $\text{C}^{14}$  remained in the trichloroacetic acid-soluble fraction at the end of the incubation period, and, from the previous study (1), would be expected to be present largely as lactic acid and unchanged glucose. No differences were found between the virus-infected and control flasks in this fraction.

Approximately 1.75 per cent of the initial radioactivity was found in the carbon dioxide formed during the experimental period with no significant difference between the control and the infected tissue. This would correspond to the complete oxidation of 0.053 mg. of the added glucose and is much less than that calculated from oxygen consumption (1).

A significant increase in the extent of incorporation of the carbon of the glucose substrate was apparent in the "protein" fraction of the brain tissue

infected with the virus as compared with the non-infected controls. The  $C^{14}$  accumulation rose from an average value of 0.7 per cent up to 1.6 per cent with overlapping in only one instance. The nature of the radioactive components of this "protein" fraction in virus-infected and uninfected tissues will be the subject of a future study.

TABLE I

*Effects of Propagation of Theiler's GD VII Virus on Incorporation of Radioactive Carbon from Labeled Glucose into Various Fractions of Minced 1 Day-Old Mouse Brain after 24 Hours of Incubation*

Per cent of added $C^{14}$ -glucose activity recovered								Per cent total recovery	
COs		Acid-soluble		Protein		Lipide		No virus	Virus
No virus	Virus	No virus	Virus	No virus	Virus	No virus	Virus		
2.36	2.10	94.02	91.00	0.70	1.50	1.10	0.58	98.18	95.18
2.50	2.00	95.98	92.11	0.62	1.20	1.02	0.62	100.12	95.93
2.60	2.01	94.20	98.59	0.68	1.41	1.08	0.72	98.56	102.73
1.30	2.09	86.22	93.00	1.00	1.98	1.30	0.78	89.82	97.85
1.40	1.40	93.78	95.55	1.11	2.11	1.40	0.90	97.69	99.96
1.30	1.51	92.00	91.95	1.19	2.07	1.36	0.90	95.85	96.43
1.20	1.29	89.65	94.00	0.48	1.93	1.04	0.41	92.37	97.63
1.60	1.40	87.95	88.63	0.52	2.00	1.10	0.49	91.17	92.52
1.40	1.23	88.00	91.37	0.50	1.23	1.05	0.50	90.95	94.33
2.40	1.17	88.30	90.40	0.59	1.27	1.45	0.70	92.74	93.54
2.70	2.36	95.40	86.90	0.70	1.20	1.40	0.59	100.20	91.05
2.68	1.94	96.90	94.20	0.61	1.10	1.50	0.41	101.69	97.65
1.76	2.63	89.45	88.30	0.80	1.47	1.35	0.40	93.36	92.80
1.70	2.37	93.55	90.60	0.59	1.53	1.35	0.60	97.19	95.10
1.90	1.69	93.00	88.92	0.81	1.60	1.41	0.43	97.12	92.64
1.50	1.81	87.62	93.18	0.78	1.45	1.39	0.33	91.29	96.77
1.70	1.50	95.68	87.30	0.52	1.45	1.62	0.44	99.52	90.69
1.70	1.80	89.00	88.11	0.60	1.70	1.18	0.80	92.48	92.41
Average 1.87 ±0.10*	1.80 ±0.10	91.15 ±0.20	91.23 ±0.20	0.71 ±0.10	1.57 ±0.10	1.28 ±0.10	0.59 ±0.10	95.57	95.29
$P = 0.553†$		$P = 0.553$		$P = 0.001$		$P = 0.001$			

\* Standard error of mean calculated from S.E.M. =  $\sqrt{(\Sigma d^2/(n-1))/n}$ .

† Calculated after Fisher for small sample statistics.

In contrast to the stimulating effect of virus propagation on the incorporation of  $C^{14}$  into the "protein" fraction, it is seen that the incorporation into the lipide fraction was significantly decreased. The decrease from 1.3 per cent to 0.6 per cent was without overlapping values. The significance of this observation is not now apparent. The effect does not cor-

respond with the increased phosphorus turnover of phospholipides noted in the previous studies with  $P^{32}$  (2).

*Incorporation of  $C^{14}$ -Labeled Bicarbonate*—The fixation of carbon dioxide into metabolizing animal tissues has frequently been demonstrated, and it was of interest to investigate the effect of Theiler's GD VII virus on such carbon dioxide fixation.

Table II shows the data for the incorporation of  $NaHC^{14}O_3$  into the various fractions of minced, 1 day-old mouse brain after 24 hours of incubation in 3 ml. of Simms' solution containing 3.03 mg. of  $C^{14}$ -labeled  $NaHCO_3$  with an activity of 0.75  $\mu$ c. per mg. In both the lipid and pro-

TABLE II

*Effects of Propagation of Theiler's GD VII Virus on Incorporation of  $NaHC^{14}O_3$  into Various Fractions of Minced One Day-Old Mouse Brain after 24 Hours of Incubation*

Per cent of initial $NaHC^{14}O_3$ activity recovered								Per cent total recovery	
CO <sub>2</sub>		Acid-soluble		Protein		Lipide			
No virus	Virus	No virus	Virus	No virus	Virus	No virus	Virus	No virus	Virus
93.50	94.00	0.46	0.66	0.14	0.21	0.36	0.24	94.46	95.11
93.11	91.59	0.42	0.70	0.19	0.23	0.27	0.34	93.99	92.86
94.64	98.62	0.53	0.49	0.21	0.18	0.41	0.22	95.79	99.51
94.00	97.53	0.47	0.51	0.15	0.16	0.34	0.33	94.96	98.53
94.30	92.37	0.56	0.39	0.12	0.15	0.33	0.35	95.31	93.26
Average... 93.91	94.82	0.49	0.55	0.16	0.19	0.34	0.30	94.90	95.85
$\pm 0.85^*$	$\pm 1.50$	$\pm 0.05$	$\pm 0.05$	$\pm 0.02$	$\pm 0.02$	$\pm 0.03$	$\pm 0.03$		
$P = 0.204^\dagger$		$P = 0.394$		$P = 0.447$		$P = 0.447$			

\* Standard error of mean calculated from  $s.e.m. = \sqrt{(\Sigma d^2/(n-1))/n}$ .

† Calculated after Fisher for sample statistics.

tein fractions the per cent of incorporation of radioactivity derived from bicarbonate was considerably less than that derived from glucose under the same conditions. No significant differences were found between the virus-infected and non-infected tissues in the amount of incorporation found in the various fractions. The extent of incorporation of  $C^{14}$  from labeled bicarbonate, though much less than that obtained with glucose, represents significant carbon dioxide fixation, since the experiments described below showed no adsorption of carbon dioxide on the various fractions.

*Adsorption*—The possibility that adsorption of radioactive acid-soluble compounds to the acid-insoluble fractions might effect the results shown

in Tables I and II was investigated. 1 day-old mouse brain was incubated for 24 hours with normal glucose; radioactive glucose was added at the termination of the incubation period and was followed by immediate fractionation. Over 99.5 per cent of the radioactivity was recovered in the acid-soluble fraction and less than 0.05 per cent of the initially added radioactivity was adsorbed by the acid-insoluble residue (R). These experiments would indicate that adsorption of labeled glucose was not a factor which would interfere with the conclusions reached.

The possibility that other radioactive acid-soluble components might have a greater adsorptive affinity than glucose for the acid-insoluble fraction (R) was investigated by neutralizing the acid-soluble fraction from flasks incubated 24 hours with radioactive glucose, adding it to minced tissues previously incubated for 24 hours in the presence of normal glucose, and fractionating immediately after mixing. Again less than 0.05 per cent of the  $C^{14}$  activity was found in the acid-insoluble residue. Radioactive  $NaHCO_3$  added to minced brain incubated for 24 hours in the presence of normal glucose followed by immediate fractionation showed virtually all of the radioactivity in the carbon dioxide fraction with less than 0.05 per cent in the trichloroacetic acid-insoluble fraction. These experiments indicate that the incorporation of radioactive carbon from labeled glucose or bicarbonate into the lipide and "protein" fractions involves little or no correction due to adsorption of acid-soluble components.

#### DISCUSSION

These data have demonstrated that Theiler's GD VII virus increases the extent of the incorporation of glucose fragments into the "protein" fraction and decreases the incorporation into the lipide fraction of minced 1 day-old mouse brain. This effect occurs during the period of maximal virus propagation (2) and may possibly be interpreted as a redirection of carbon metabolism in the virus-infected tissue.

It has previously been reported (2) that during the period of maximal virus propagation, the uptake of  $P^{32}$  into the phospholipide and ribose nucleic acid phosphate fractions was stimulated. The present  $C^{14}$  data show that the virus stimulates the incorporation of glucose fragments into the "protein" fraction but inhibits their incorporation into the lipide fraction of the host tissue. The discrepancy between the  $C^{14}$  and  $P^{32}$  data concerning the effect of virus propagation on lipide metabolism of minced mouse brain tissues may be more apparent than real, since the different components of the phospholipide molecule may turn over at different rates. Likewise, the uptake of  $P^{32}$  is an indication of the metabolic activity of the phospholipides only, whereas the incorporation of glucose fragments may be a measure of the metabolic activity of other tissue lipides as well.



The relatively small amount of carbon dioxide fixation into "protein" and lipid fractions observed in these experiments and the failure of virus propagation to influence the extent of carbon dioxide fixation suggest that this process may not play a significant rôle in the metabolic processes concerned with virus propagation, or that a low level of fixation fulfils the requirements.

It will be noted in Table I that about 96 per cent of the initial radioactivity was recoverable in the various fractions. Another 1 to 3 per cent in the various washes which were discarded. This still left 2 to 4 per cent of the initial radioactivity not accounted for. Although a perfect balance is highly desirable for isotopic studies, it is felt that this loss is close to the experimental error of our methods and should not impair the conclusions reached.

#### SUMMARY

The effects of the propagation of Theiler's GD VII strain of mouse encephalomyelitis virus on the incorporation of radioactive carbon from labeled glucose into the tissue components of minced, 1 day-old mouse brain incubated for 24 hours at 35° have been studied. During the period of maximal virus propagation there is an increased incorporation of glucose fragments into the "protein" fraction and a decreased incorporation into the lipid fraction of virus-infected tissue, as compared with non-infected minces. Virus propagation had no influence on the relatively slower fixation of radioactive carbon dioxide into lipid and "protein" fractions.

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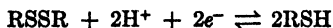
# OXIDATION-REDUCTION POTENTIALS OF THIOL-DISULFIDE SYSTEMS\*

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The importance of sulfhydryl compounds in biological systems has led to considerable interest in the following equilibrium.



Dixon and Quastel (1) were the first to attempt to measure the oxidation-reduction potential of this reaction. Their data fit an equation of this form,

$$\pi = \pi_0 + \frac{RT}{F} \ln [\text{H}^+] - \frac{RT}{F} \ln c$$

where  $\pi$  is the observed E.M.F.,  $\pi_0$  is an undefined "normal reduction potential," and  $c$  is the concentration of thiol.

Kendall and Nord (2) reported that a trace of hydrogen peroxide, sodium disulfide, or molecular oxygen enables cysteine to reduce indigo-carmin and makes it possible for cystine to oxidize reduced indigo. From evidence of this type, they concluded that the thiol-disulfide system is reversible. Dixon and Tunnicliffe (3) objected to this deduction; they stated that even in the absence of any catalyst cysteine reduces indigo-carmin. Kendall and Loewen (4) could not confirm this observation. They found that in the presence of a constant concentration of the "activating agent" the velocity of oxidation and reduction is influenced by the ratio of thiol to disulfide; they reported, also, that in the presence of indigo and "catalyst" the disulfide concentration affects the potential of a solution of cysteine. Whatever the merits of this controversy, it was not shown that mixtures of thiol and disulfide establish potentials which are in accord with the appropriate thermodynamic equations.

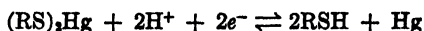
Dixon (5) proposed that the potential of a thiol solution is determined by an equilibrium in which the rate of transference of hydrogen atoms to the electrode is balanced by the diffusion of hydrogen gas from the electrode. Harrison and Quastel (6) showed that the potential of a metal-free cysteine solution is not affected by the addition of small amounts of

\* This paper is from the doctoral dissertation of Leon D. Freedman, The Johns Hopkins University, 1949.

† Predoctorate Fellow of the National Institutes of Health, 1949.

ferric or cupric ions. They believed that this result disproved the mechanism that Dixon had advanced.

Michaelis and Flexner (7) confirmed the electrode equation deduced by Dixon and Quastel, and showed that all the proposed explanations were inadequate. Barron, Flexner, and Michaelis (8) suggested that the cysteine potential at the mercury electrode is due to the following reaction.



Under fixed experimental conditions, they said, the concentration of mercuric cysteinate remains constant and is unaffected by the ratio of thiol to disulfide or the pH. They presented little quantitative evidence for this theory, but they did show that metallic mercury is attacked by cysteine to form slightly soluble complexes.

Williams and Drissen (9) and Fischer (10) attempted to solve the problem by potentiometric titration of the reduced form. They obtained different  $E_0$  values with different oxidizing agents. In the case of titrations with iodine, they found that the potential changed approximately 30 mv. per pH unit instead of the theoretical 59. Nevertheless, they calculated a "normal potential" by arbitrarily using the equation

$$E_0 = E_{\text{obs.}} - 0.059 \log \frac{[\text{RSSR}]^{\frac{1}{2}}}{[\text{RSH}]} + 0.030 \text{ pH}$$

Ghosh and his coworkers (11-13) reported that it is possible to obtain a reversible thiol-disulfide system by the partial reduction of buffered solutions of disulfide at a mercury cathode. Their procedure, they stated, removes an oxide film from the mercury and thus enables it to function as a truly inert electrode. They reported the same  $E_0$  values for cysteine, thioglycolic acid, and thiolactic acid; from this evidence, they concluded that the force between the sulfur and hydrogen atoms is independent of the rest of the molecule. This deduction cannot be correct, for the pK values of thiols vary over a considerable range (14-19).

Green (20) was able to repeat the experimental results of Ghosh and his coworkers. He believed, however, that a mercury-thiol complex (which he did not show was present) was formed in the electrolysis, and that this complex made the thiol-disulfide system electromotively active. He stated, too, that in the absence of oxygen and in the absence of any electric current such complexes were not present; therefore, he could not accept the theory of Barron, Flexner, and Michaelis (8).

In a paper which is not mentioned by later investigators, Larsson (21) reported that the potentials of the thioglycolic acid-dithiodiglycolic acid system are made erratic by traces of iron or copper salts. In solutions

which contained  $10^{-7}$  mole per liter of these ions, Larsson found a rapid adjustment of the observed potentials to values which could be interpreted by the conventional equations.

Fruton and Clarke (22) used dyes of known potential in an attempt to measure the  $E_0$  values of several compounds containing sulfhydryl groups. Hellerman (23) has pointed out that the versatile reactivity of thiols makes the assumed reaction between dye and mercaptan questionable.

Borsook and his coworkers (24) calculated the standard electrode potentials of several thiol-disulfide systems from thermal measurements. Their method is certainly valid, but experimental difficulties prevent the attainment of high accuracy.

Kolthoff and Barnum (25) studied the polarographic behavior of cysteine. Their results showed that cysteine is not oxidized to cystine at the dropping mercury electrode. Normal anodic waves were not obtained with the platinum micro wire electrode, nor were they obtained with the mercury electrode except at pH 1. Analysis of the normal wave indicated that it could be governed by the reaction



Since no compound has ever been isolated in which mercurous ion is present as  $\text{Hg}^+$ , it seems unlikely that this mechanism is correct. Cartledge (26) has found that the equilibrium constant for the conversion of  $\text{Hg}^+$  to  $\text{Hg}_2^+$  is about  $10^{31}$ .

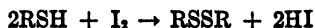
Kolthoff and Barnum (27) have also described the reduction of cystine at the dropping mercury electrode. The waves obtained were very complex; however, there is little doubt that the potentials observed do not refer to the thiol-disulfide system.

Ryklan and Schmidt (28) reported that iodide ion catalyzes the cystine-cysteine equilibrium. Using iodine or potassium permanganate as oxidizing agent, they determined the normal potentials of eight thiols.

If the results reported by Ryklan and Schmidt are valid, then the problem is solved, for the method they describe is simple and rapid. However, there are several reasons to doubt that the potentials they observed were determined by the thiol-disulfide equilibrium. First of all, it is well known that iodine can react with thiols to form compounds other than disulfides. For example, Lucas and King (29) have shown that the amount of iodine consumed in the titration of cysteine varies with the temperature, pH, initial concentration of thiol, iodide concentration, and the strength of the iodine solution used.

Some of the experiments described by Ryklan and Schmidt are rather puzzling. For their potentiometric titrations they mixed 10 ml. of a

0.05 M buffer solution with an equal volume of 0.05 M thiol solution. Then they titrated with an oxidizing agent, usually iodine. Assuming that the stoichiometric equation is



the solution must become more acid during the titration (if the original pH of the buffer-thiol mixture is greater than about 1.4). In the absence of a constant pH the results become difficult to interpret.

The earlier work of Williams and Drissen (9) and Fischer (10) indicated that iodine titrations do not yield potentials which obey the thermodynamic formulations. It seems strange that Rykkan and Schmidt should obtain such different results.

Rykkan and Schmidt report also that cystine affects the potentials of cysteine solutions even in the absence of iodide ion or other catalyst. This result differs from the observations reported by several earlier investigators (1, 2, 7).

Recently Preisler and his coworkers have reported that the thiourea-formamidine disulfide system (30) and the dithiobiuret-3,5-diimino-1,2,4-dithiazoline system (31) are reversible. He has indicated (32) also that he has been unable to confirm the conclusions of Rykkan and Schmidt.

#### *Materials and Methods*

The titration vessel used was similar to the one described by Michaelis and Eagle (33). In the experiments involving electrolytic reduction, a cell like that employed by Ghosh, Raychaudhuri, and Ganguli (11) was used. Nitrogen gas was purified by passing it through a tube packed with copper wire which was heated to about 500°. The reference half-cell used in all the experiments was a saturated calomel electrode; the potential of this electrode was taken from Clark (34). The two half-cells were joined by an agar-KCl bridge. Liquid junction potentials were ignored. The thermostat used was an oil bath, the temperature of which could be controlled to  $\pm 0.02^\circ$ . The electrode potentials were determined with a Leeds and Northrup student potentiometer, which could be read to  $\pm 0.1$  mv.

All solutions used were shown to contain less than 1 part in 10 million of either iron or copper. The color test described by Lyons (35) was used to estimate the concentration of iron, and the dithizone method was used to show the virtual absence of copper.

All pH measurements were made with a glass electrode in conjunction with a Beckman model G pH meter.

Thioglycolic acid was purified by vacuum distillation. It boiled at

101–103° at 11 mm. Dithiodiglycolic acid was prepared by the method of Harrison (36); m.p. 106–107°.

Cysteine hydrochloride was obtained from the Pfanstiehl Chemical Company. Analysis showed that it contained 1 mole of water per mole of cysteine.

The L-cystine supplied by the Eastman Kodak Company was purified by solution in dilute alkali and precipitation with acetic acid.

The thiourea was recrystallized from hot water. Formamidine disulfide was prepared by the method of Präisler and Berger (30).

A sample of ethylene thiourea which was kindly furnished us by the Rohm and Haas Company was purified by recrystallization from hot water.

The method used to analyze solutions of cysteine and thioglycolic acid was based on the work of Lavine (37).

The determination of traces of mercury was carried out by means of the dithizone method outlined by Sandell (38).

The semimicrodetermination of mercury was performed by the method of Bartlett and McNabb (39).

The mercury used for electrodes was purified by the method described by Wichers (40).

#### RESULTS AND DISCUSSION

*Iodide Ion As Mediator*—Using thioglycolic acid, we have attempted to repeat some of the work of Rykkan and Schmidt (28). In order to keep the pH constant during oxidations with iodine, standardized sodium hydroxide solution was added (when necessary) as the titration proceeded. If this was not done, we found that large changes in pH occurred during the titration of solutions, the initial pH of which was greater than about 1.4. For example, when a buffer-thiol mixture of pH 2.4 was titrated with iodine alone, the pH at the end-point was 1.9. Of course, we could have used higher buffer to thiol ratios instead of adding sodium hydroxide. However, we preferred to use solutions of the same ionic strength as those employed by Rykkan and Schmidt.

At a constant pH, the electrode equation assumed by Rykkan and Schmidt can be reduced to

$$E_{\text{obs.}} = \text{"constant"} + \frac{RT}{F} \ln \frac{x^{\frac{1}{2}}}{100 - x} + \frac{RT}{2F} \ln \frac{V_0 + \Delta V}{V_0}$$

where  $x$  is the percentage oxidation,  $V_0$  is the original volume of the solution being titrated, and  $\Delta V$  is the increase in volume due to the addition of the iodine solution and the sodium hydroxide solution.

The curves in Fig. 1 were drawn from points calculated with the aid of the above equation. The "constant" in the equation was the average

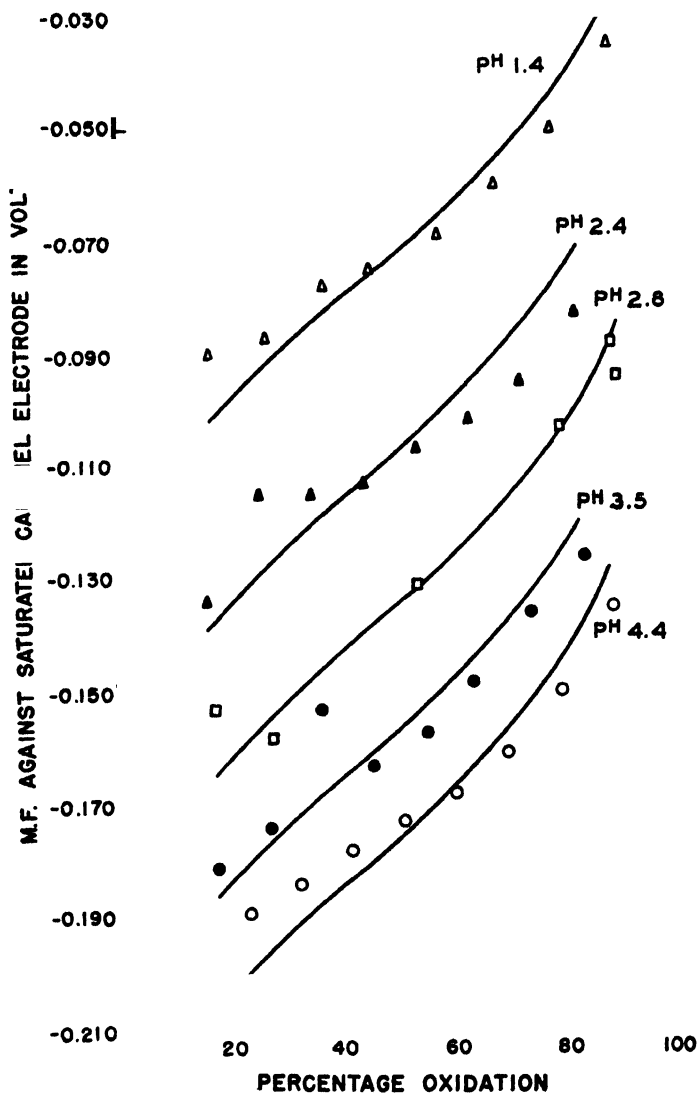


FIG. 1. Potentials observed with the platinum electrode

of values obtained by substituting the potentials observed with a platinum electrode between 10 and 90 per cent oxidation. It is immediately apparent that the assumed electrode equation is not closely obeyed.

Other anomalous results were obtained. It was very difficult to obtain precise readings with a platinum wire electrode. The galvanometer seemed very sluggish and insensitive; an "off balance" of 1 mv. produced a barely noticeable deflection. This observation suggests the possibility that a platinum-thiol compound is formed on the surface of the electrode. Such a film would be expected to increase the resistance of the cell.

It was strange, too, that the change in potential from 10 to 90 per cent oxidation was always less than theoretical. In no case was the difference between the observed and calculated change within the limits of experimental error.

Potentiometric measurements were made also with the boron carbide electrode. This electrode in conjunction with quinhydrone has been successfully used for the measurement of the ionization constants of a number of acid-substituted mercurials (41).

The most significant fact about the thiol-disulfide potentials observed with the boron carbide electrode is that they were always different from those determined with the platinum electrode. The fact, alone, casts doubt on the idea that iodide ion promotes reversibility of the thiol-disulfide system. In well poised iodine-iodide solutions, boron carbide yields the same potentials (to within 1 mv.) as does platinum.

It is difficult to generalize further about the sulfhydryl potentials recorded by boron carbide for the reproducibility of such measurements was poor. In no case, however, did the observed potentials even approximate those of the assumed electrode equation.

In addition to the discrepancies already mentioned, we found that even in the presence of iodide ion dithiodiglycolic acid did not affect the potential nearly as much as theory predicts. For example, in one experiment we had a titration vessel containing 22.03 ml. of solution of the following composition:  $[RSH] = 0.0164$ ;  $[RSSR] = 0.0029$ ;  $[I^-] = 0.25$ ;  $pH = 1.4$ . The potential of a platinum wire electrode immersed in the solution was  $-0.084$  volt (against the saturated calomel electrode); that of a boron carbide electrode was  $+0.133$  volt (against the saturated calomel electrode). To this solution was added 89.5 mg. of dithiodiglycolic acid. This increased the disulfide concentration to  $0.0252$  M. Therefore, the potential should have increased by an amount equal to  $0.030 \log (0.0252/0.0029)$  or  $0.027$  volt. Actually we found that the E.M.F. of the platinum electrode had become  $-0.079$  volt (an increase of only  $0.005$  volt) and that the potential of the boron carbide electrode had decreased to  $+0.126$  volt.

From the work described above, it appears that the potentials of platinum or boron carbide electrodes immersed in solutions of thioglycolic acid and dithiodiglycolic acid do not refer to the thiol-disulfide system.

*Cysteine Potential at Mercury Electrode*—When we became convinced that the results of Rykjan and Schmidt (28) do not represent valid measure-



ments of thiol-disulfide potentials, we decided to investigate the method of Ghosh and his coworkers (11-13).

By using an Esterline-Angus recording milliammeter in some of the electrolysis experiments, we found that cystine at pH 9 can be reduced with virtually 100 per cent current efficiency (Table I). This fact enabled us to simplify the method used by Ghosh. By measuring the current consumption, we could reduce a known fraction of the cystine, cut off the applied voltage, and wait for equilibrium. It was no longer necessary to withdraw samples for analysis. When we used 0.1 M borate buffer as the solvent, we found that the change in pH from 0 to over 90 per cent reduction was less than 0.04.

The procedure just described made it possible to keep the volume of the solution constant and obtain points from a few per cent reduction to almost 100 per cent reduction. In other words we were able to "titrate" the disulfide with electrons. Using this method we obtained the curve shown in Fig. 2. A considerable number of similar experiments left no

TABLE I  
*Comparison of Current Consumption and Disulfide Reduction*

Average current	Duration of electrolysis	Faradays used	Thiol found
ma.	min.		mole
0.91	405	$2.30 \times 10^{-4}$	$2.31 \times 10^{-4}$
0.90	300	$1.68 \times 10^{-4}$	$1.65 \times 10^{-4}$
0.90	45	$0.25 \times 10^{-4}$	$0.24 \times 10^{-4}$

doubt that the electrode equation assumed by Ghosh is obeyed more closely than the semiempirical relationship proposed by Dixon and Quastel (1).

These results do not, however, tell us whether cystine affects the electrode potential. This question was answered by performing the following experiments. In one experiment 25 ml. of 0.01 N (*i.e.* 0.005 M) cystine buffered with borate at pH 9.0 were placed in the titration vessel and reduced 10 per cent; *i.e.* the resulting solution contained 0.001 N cysteine and 0.009 N cystine. The "equilibrium" potential observed was -0.586 volt (against the saturated calomel electrode).

In a second experiment an equal volume of 0.002 N cystine similarly buffered was placed in the electrode vessel, where 50 per cent of it was reduced; *i.e.* the resulting solution contained 0.001 N cysteine and 0.001 N cystine. Since this second solution contained only one-ninth as much cystine as the first solution and since both solutions contained an equal concentration of cysteine, we would expect the potential in the second experiment to be more negative by an amount equal to 0.030 log 9/1 or

0.029 volt. Actually the second solution gave a potential of  $-0.566$  volt, a value more positive than the E.M.F. observed in the first experiment.

Apparently, then, the method of Ghosh does not yield potentials which are characteristic of thiol-disulfide systems. If this conclusion is correct, then what oxidation-reduction system is responsible for the values ob-

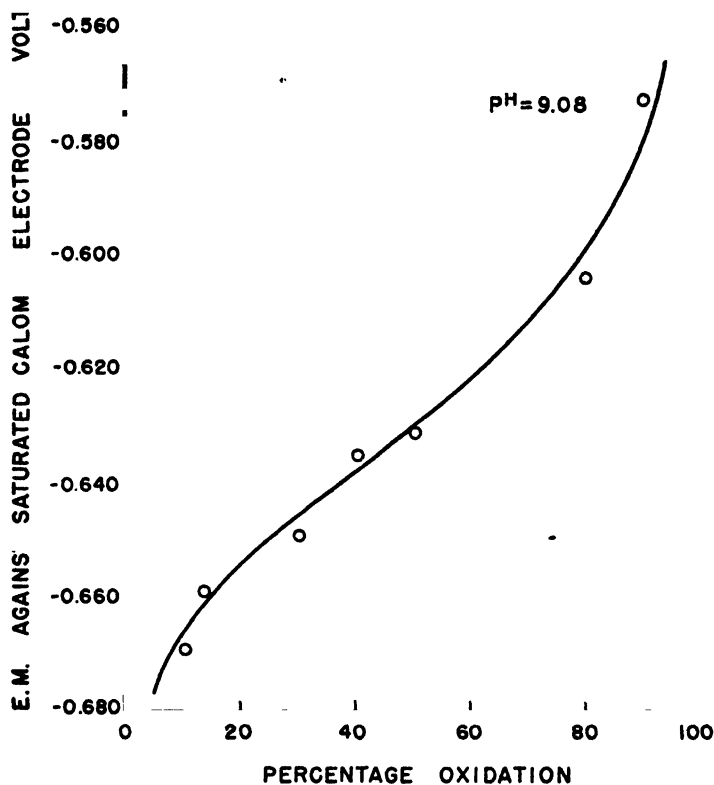
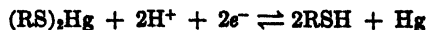


FIG. 2. Potentials obtained by the method of Ghosh; temperature  $30^{\circ}$

tained? Barron, Flexner, and Michaelis (8) had proposed that the cysteine potential at the mercury electrode is due to the following reaction.



Ghosh and his coworkers were aware of this suggestion. They believed, however, that after electrolytic reduction there is no mercury complex dissolved in the solution, since it fails to give a precipitate with hydrogen sulfide. Their conclusion was, therefore, that the cysteine-mercuric cysteinate system could not be involved in their measurements.

We confirmed the observation of Ghosh (and of Green) that solutions

of disulfide after electrolysis at a mercury cathode do not give precipitates with hydrogen sulfide. This fact does not prove, however, that such solutions are completely free of mercury; solutions of cysteine to which small amounts of mercuric chloride were added gave no immediately visible precipitates with hydrogen sulfide unless the concentration of mercury exceeded  $2 \times 10^{-5}$  M.

Using the dithizone method (38), we learned that thiol-disulfide solutions prepared by the method of Ghosh do contain divalent mercury. Furthermore, we found that the concentration of mercury varies roughly in accordance with the electrode equation proposed by Barron, Flexner, and Michaelis (8). This is shown by some data in Table II.

We cannot expect the values of  $E_0$  from these experiments to agree any more closely. For not only do we have the usual errors due to uncertainties in pH, in analytically determined concentrations, and in observed E.M.F., but in addition these potentials are very poorly "poised," since in these

TABLE II  
*Mercury in Solution after Electrolytic Reduction of Cystine*

pH	Cysteine	Cystine	Mercury	E.M.F.*	$E_0$ †
	M	M	M	volt	volt
9.05	0.00100	0.00450	$2.3 \times 10^{-6}$	-0.586	0.188
9.02	0.00100	0.00050	$19.6 \times 10^{-6}$	-0.566	0.178

\* E.M.F. versus saturated calomel electrode; temperature, 30°.

† These values were calculated on the assumption that the observed potentials are governed by the equilibrium,  $(RS)_2Hg + 2H^+ + 2e^- \rightleftharpoons Hg + 2RSH$ .

experiments the cysteine-mercuric cysteinate system is almost entirely in the reduced state. Qualitatively, however, there is little question that cystine cannot be the potential-determining oxidant and that mercuric cysteinate may be.

Another set of observations led us to the same conclusion. In these experiments, 10 ml. of a buffered solution of 0.01 N cystine were placed in the electrode vessel, deaerated, and then electrolyzed at a potential not quite negative enough to reduce a detectable amount of disulfide. Then 0.01 N cysteine solution was added. After the potential had become constant, additional 0.01 N cystine solution was introduced into the titration vessel. The change in potential caused by the additional cystine solution could conceivably be governed by one of three electrode equations, (1) the thiol-disulfide relationship assumed by Ghosh, (2) the relationship proposed by Dixon and Quastel, or (3) the cysteine-mercuric cysteinate relationship. If any one of these three possibilities holds, we would expect

the potential to become more positive after the further addition of the cystine solution. However, the magnitude of the change is appreciably different for each case. If the thiol-disulfide system is the potential-determining system, then we would expect the observed E.M.F. to increase by an amount equal to the difference between  $0.060 \log (\text{final } [\text{RSSR}]^{\dagger})/(\text{final } [\text{RSH}])$  and  $0.060 \log (\text{initial } [\text{RSSR}]^{\dagger})/(\text{initial } [\text{RSH}])$ . If the electrode equation of Dixon and Quastel applies, then we would expect the potential to increase by an amount equal to  $0.060 \log (\text{initial } [\text{RSH}])/(\text{final } [\text{RSH}])$ . If the cysteine-mercuric cysteinate system is the potential-determining system, then we would expect an effect due only to dilution of the solution; this change would be equal to  $0.030 \log (\text{final volume})/(\text{initial volume})$ . The results presented in Table III show clearly that the potential is not governed by either relationship (1) or (2) and that it may be determined by the cysteine-mercuric cysteinate system.

TABLE III  
*Comparison of Observed and Theoretical Potential Changes*

pH	Initial volume	Final volume	$\Delta E$ calculated from*			$\Delta E$ found
			Relation- ship (1)	Relation- ship (2)	Relation- ship (3)	
	ml.	ml.	mv.	mv.	mv.	mv.
9.05	34.00	59.00	24	14	7	6
1.12	35.00	55.00	21	12	6	8

\* See the text.

Compounds of mercury and cysteine have been reported by a number of investigators (42-46). We prepared mercuric cysteinate by mixing a solution of 1.7 gm. of cysteine hydrochloride in 25 ml. of water with a solution of 1.4 gm. of mercuric chloride in 25 ml. of water. In a few minutes a white, crystalline material began to come out of the solution. After several hours in the ice box, the compound was filtered, washed with cold water, and dried over sodium hydroxide pellets. A yield of 1.9 gm. was obtained. Analysis of this material indicated that it is a mixture of the free base and the hydrochloride of mercuric cysteinate. There is no question that it contains 1 mercury atom to 2 cysteine molecules.

*Analysis—*

$\text{C}_2\text{H}_4\text{Cl}_2\text{HgN}_2\text{O}_4\text{S}_2$ .	Calculated.	C 14.05, H 2.76, Cl 13.83, Hg 39.04, N 5.46
$\text{C}_2\text{H}_4\text{HgN}_2\text{O}_4\text{S}_2$ .	"	" 16.33, " 2.74, " 45.48, " 6.85
Both compounds.	Found.	" 14.74, " 2.72, " 8.45, " 40.32, " 5.68
	Calculated.	C:Hg 6.00, N:Hg 2.00
	Found.	" 6.11, " 2.02

We found that dithizone in carbon tetrachloride extracts mercury quantitatively from dilute, acid solutions of mercuric cysteinate. Some typical analyses on 10 ml. samples are given in Table IV.

These results are pertinent not only to the use of the dithizone method for determining the concentration of mercuric cysteinate, but also to the use of dithizone derivatives as antidotes for heavy metal poisoning (47).

It was of considerable interest to determine whether mixtures of cysteine and mercuric cysteinate would yield potentials which obeyed the requirements of a reversible system and which were consistent with the  $E_0$  values in Table II. Our experimental results leave no question that such is the

TABLE IV  
*Recovery of Mercury from Mercuric Cysteinate Solutions*

Mercury taken	Mercury found
$\gamma$	$\gamma$
12.0	12.3
10.0	10.3
10.0	9.7

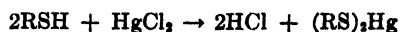
TABLE V  
*Potentials of Cysteine-Mercuric Cysteinate Mixtures*

RSH	(RS) <sub>2</sub> Hg	E.M.F.*	$E_0$
<i>M</i>	<i>M</i>	volt	volt
0.00500	0.00262	-0.065	0.185
0.00600	0.00210	-0.074	0.184
0.00655	0.00181	-0.079	0.183
0.00714	0.00151	-0.082	0.185

\* E.M.F. versus saturated calomel electrode; temperature, 30°.

case. Some data obtained with solutions in 0.1 N hydrochloric acid (pH = 1.15) are presented in Table V.

When solutions of cysteine of known concentration were placed in the mercury electrode vessel and titrated potentiometrically with standardized mercuric chloride, the end-point appeared a little before the value expected from the equation



We interpreted this apparently premature end-point to mean that the thiol plus traces of oxygen in the solution had reacted with the mercury electrode to form a small amount of mercuric cysteinate. This explanation seems reasonable in the light of the work of Michaelis and Barron (48).

The results of a titration of 25 ml. of 0.00931 M cysteine in 0.1 N HCl (pH = 1.10) with 0.00500 M mercuric chloride in a similar buffer are shown graphically in Fig. 3. A plot of  $\Delta E$  versus  $\Delta$  ml. located the end-point at 17.90 ml. of the mercuric chloride solution. Since the expected end-point was 18.62 ml., we calculated that 0.00017 M (RS)<sub>2</sub>Hg was present in the cysteine solution before any mercuric chloride was added. On this as-

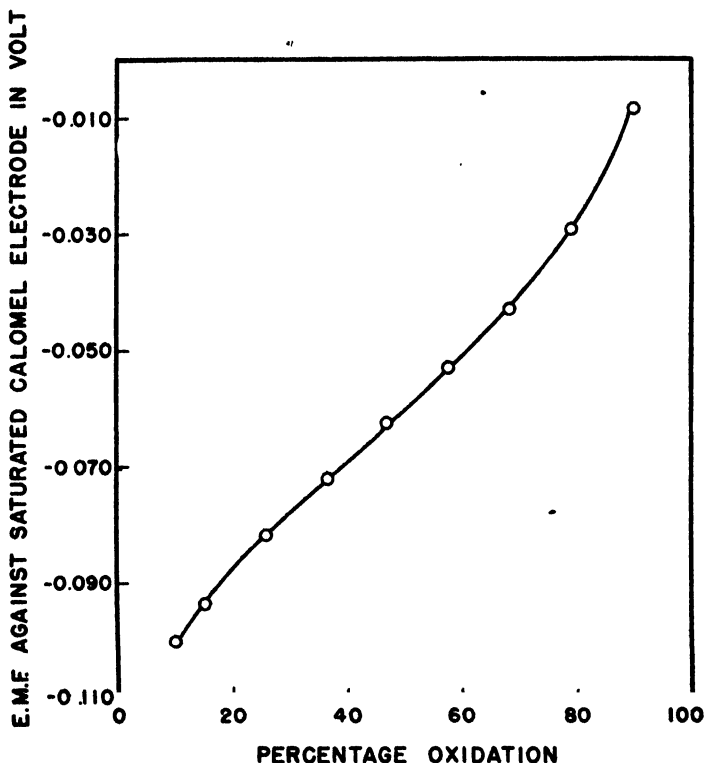


FIG. 3. Titration of cysteine with mercuric chloride; temperature 30°

sumption, the value of  $E_0$  proves to be 0.182 volt, which differs but little from the values shown in Table V.

Similar results were obtained by the following procedure. Exactly 10 ml. of buffer solution were placed in the mercury electrode vessel and subjected to electrolytic reduction at a potential not quite negative enough to liberate hydrogen. When the current became less than  $10^{-5}$  ampere, the applied voltage was removed. Then a small quantity of standardized mercuric chloride solution was introduced into the cell. Finally, varying quantities of cysteine solution were added from a burette. The first

portion of the cysteine solution added was in excess of that needed to bind completely the mercuric chloride present. After each addition of cysteine, the potential, which became constant in about 3 minutes, was recorded. In this way, potentials were obtained under conditions in which the concentration of thiol was large compared to the concentration

TABLE VI  
*Potentials of Mixtures of Cysteine and Mercuric Chloride*

RSH	(RS) <sub>2</sub> Hg	E.M.F.* at pH 8.97	E <sub>0</sub>
<i>M</i>	<i>M</i>	<i>volt</i>	<i>volt</i>
0.00074	0.000040	-0.5402	0.185
0.00144	0.000037	-0.5584	0.185
0.00203	0.000035	-0.5685	0.185
0.00256	0.000032	-0.5751	0.185
0.00300	0.000030	-0.5803	0.185
0.00375	0.000027	-0.5872	0.185
0.00461	0.000023	-0.5952	0.185
0.00616	0.000016	-0.6066	0.186
0.00675	0.000013	-0.6113	0.186

\* E.M.F. versus the saturated calomel electrode; temperature, 30°.

TABLE VII  
*Potentials of Mixtures of Cysteine and Mercuric Chloride*

RSH	(RS) <sub>2</sub> Hg	E.M.F.* at pH 8.99	E <sub>0</sub>
<i>M</i>	<i>M</i>	<i>volt</i>	<i>volt</i>
0.00059	0.000076	-0.5288	0.183
0.00123	0.000070	-0.5483	0.184
0.00179	0.000065	-0.5589	0.184
0.00228	0.000061	-0.5658	0.184
0.00271	0.000057	-0.5710	0.185
0.00309	0.000054	-0.5753	0.184
0.00343	0.000051	-0.5788	0.184
0.00424	0.000044	-0.5862	0.184
0.00521	0.000036	-0.5946	0.184
0.00575	0.000031	-0.5983	0.185
0.00635	0.000026	-0.6034	0.185

\* E.M.F. versus the saturated calomel electrode; temperature, 30°.

of mercuric cysteinate. Some data obtained by this technique are shown in Tables VI and VII.

By combining the potential of the mercury-mercuric couple given by Latimer (49) with the potential of the cysteine-mercuric cysteinate couple, we can calculate the instability constant of mercuric cysteinate. Its

value is about  $5 \times 10^{-2}$ . This shows that mercuric cysteinate is more stable than  $\text{HgCl}_4^-$  or  $\text{HgBr}_4^-$ , but at pH 0 less stable than  $\text{HgI}_4^-$  (50). In 1 N hydriodic acid, 1 mole of mercuric cysteinate consumes 2 equivalents of iodine. As should be expected, too, the nitroprusside test for the free sulfhydryl group is negative with mercuric cysteinate.

The experiments and calculations discussed in this section make it possible to give a simple, consistent explanation of the observations recorded in the literature of cysteine-potentials at mercury electrodes. Many of the data reported by Barron, Flexner, and Michaelis (8) are in disagreement with their hypothesis that such potentials are governed by the cysteine-mercuric cysteinate equilibrium. They ascribe this lack of agreement to the "formation of higher complexes" and possibly activity corrections. We believe that the most important reason that the changes in potential they observed were smaller than expected is that the mercury electrode reacted with the cysteine to form a significant quantity of mercuric cysteinate. In similar experiments we have found that the concentration of mercuric cysteinate formed by the reaction between thiol and electrode may be as high as  $2 \times 10^{-4}$  M. Therefore, we cannot neglect the initial concentration of mercuric cysteinate and expect the potential to depend only on the amount of mercury added.

Green (20) describes an experiment in which 0.1 M cysteine buffered at pH 7.35 produced a potential of  $-0.281$  volt at a mercury electrode. After electrolytic reduction the potential changed to  $-0.305$  volt. He states, "The only plausible explanation is that the mercury electrode suffered some change." We believe that a more likely reason for the change in potential is that the electrolysis decreased the concentration of mercuric cysteinate present. Our experiments show that mercuric cysteinate can be reduced electrolytically to cysteine and metallic mercury.

All of the evidence discussed in this paper leaves little doubt that mercuric cysteinate was the potential-determining oxidant present in the experiments of Ghosh. An additional inference that may be drawn is that the following equilibrium is not rapidly reversible:



for if this reaction were able to take place rapidly, then the mercury complex in low concentration could "mediate" thiol-disulfide systems. This is apparently not the case.

*Thiourea-Formamidine Disulfide and Related Systems*—Our observations on the thiourea-formamidine disulfide system are in complete agreement with those of Preisler and Berger (30). We found, however, that electrodes of boron carbide or gold-plated platinum do not yield potentials which are in accord with the theoretical electrode equation. This is illustrated



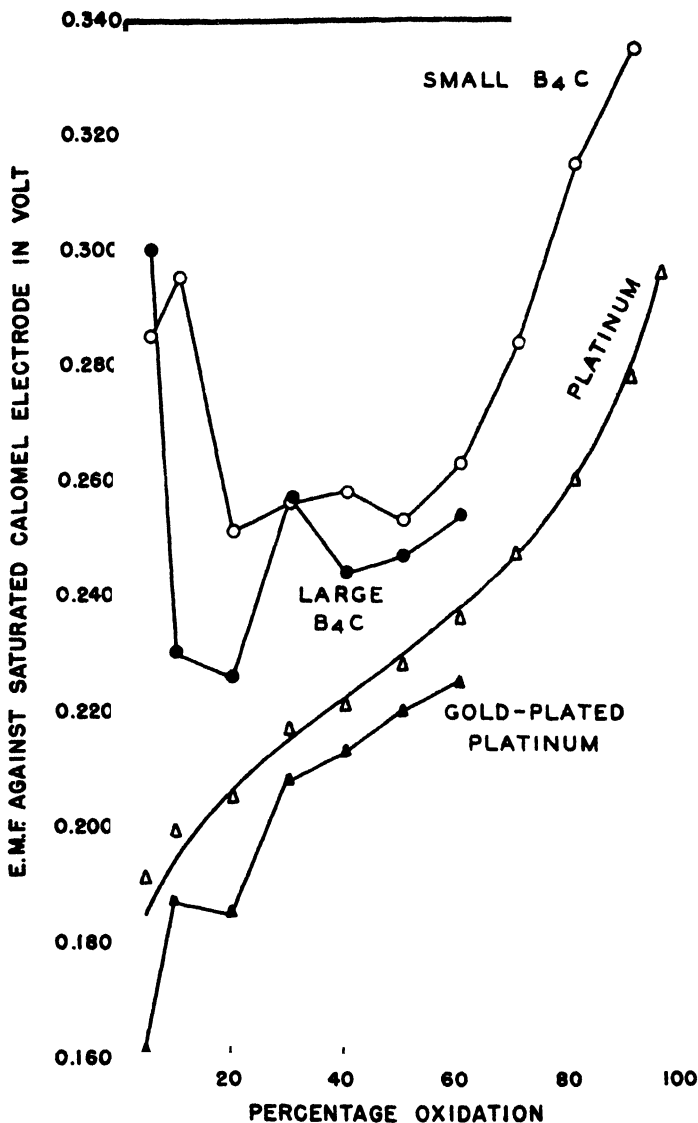


Fig. 4. Potentials of the thiourea-formamidine disulfide system; temperature 25°

in Fig. 4. These results may indicate that even this thiol-disulfide system is not completely electromotively active, but depends on some unknown reaction with the platinum electrode.

We have also investigated ethylene thiourea. The disulfide of this compound has not been reported except as an iodine complex of undeter-

mined structure (51). We prepared the disulfide of ethylene thiourea by the following procedure.

5 gm. of recrystallized ethylene thiourea were suspended in 40 ml. of dry methyl alcohol which was 4.4 N in hydrogen chloride. The mixture was cooled to 0° and stirred vigorously as 30 per cent hydrogen peroxide was slowly added. When 3.2 ml. had been added, the supernatant solution gave a negative test with the copper sulfate-hydrochloric acid reagent of Edens and Johnson (52). The suspension was allowed to remain in the ice bath for about an hour after the reaction was completed, and then was filtered. The oxidation product, which was a fine, white powder, was washed with a little dry alcohol and dried *in vacuo* over sodium hydroxide. The yield was 5.6 gm.

*Analysis*— $C_2H_{11}Cl_2N_2S_2$ . Calculated. C 26.19, H 4.36, N 20.36  
Found. " 25.91, " 4.53, " 20.71

This compound is only slightly soluble in absolute alcohol and practically insoluble in ether. It is very soluble in water or 95 per cent ethanol. When dilute iodine was added to an aqueous solution, a dark red, crystalline precipitate was formed. This material was filtered off, washed with water, and dried; m.p. 118–120° (with decomposition). Johnson and Edens (51) reported a melting point of 119° for their iodine complex.

Even in the solid state, the disulfide dihydrochloride of ethylene thiourea is rather unstable. After standing at room temperature for several months, it turned yellow and gave off the odor of hydrogen chloride. Sulfur and ethylene thiourea were identified among the decomposition products. In 1 N sulfuric acid solution, the disulfide decomposes in a few hours.

Titration of 0.01 N ethylene thiourea in 1 N sulfuric acid with 0.025 N ceric sulfate gave the results shown in Fig. 5. It was not possible to estimate the end-point potentiometrically. Platinum electrodes recorded potentials which closely obeyed the theoretical electrode equation for the first 70 per cent of the oxidation; then, apparently, the disulfide formed in the reaction begins to react with the ceric sulfate. The value for  $E_0$  calculated from the points between 5 and 70 per cent oxidation is 0.489 volt (at 25°).

Similar results were obtained by the method of mixtures. The total concentration of thiol plus disulfide was kept constant at 0.01 N; the solvent was 1 N sulfuric acid. The instability of the disulfide made it necessary to complete these experiments as soon as possible after the solutions were prepared. The data obtained are shown in Table VIII.

The potentials of thiol-disulfide systems at a constant pH should vary not only with the ratio of oxidized to reduced form but also with the total

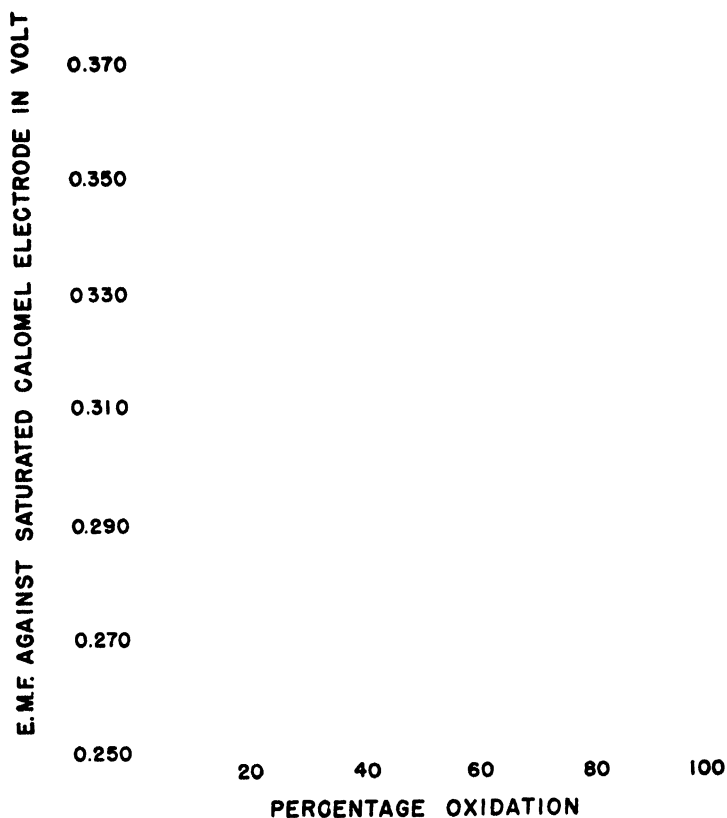


Fig. 5. Titration of ethylene thiourea with ceric sulfate; temperature 25°

TABLE VIII

*Potentials of Mixtures of Ethylene Thiourea and Its Disulfide*

Oxidation	E.M.F.*	$E_0$
<i>per cent</i>	<i>volt</i>	<i>volt</i>
28.6	0.2970	0.496
34.5	0.3022	0.496
40.0	0.3071	0.497
50.0	0.3145	0.497
58.9	0.3220	0.497
66.7	0.3301	0.498
77.0	0.3407	0.497
83.3	0.3500	0.497

\* E.M.F. versus saturated calomel electrode; temperature, 30°.

concentration. If the ratio of thiol to disulfide is kept constant and only the total concentration varied, the observed E.M.F. should obey the following relationship,  $E_{\text{obs.}} \propto 0.030 \log m$ , where  $m$  is the total concentration (in equivalents) of thiol plus disulfide.

This equation was tested by the following experiment. Exactly 10 ml. of 1 N sulfuric acid were placed in the titration cell. Purified nitrogen was bubbled through the solution for 45 minutes. Then we added varying quantities of a 1 N sulfuric acid solution which was 0.005 N in ethylene

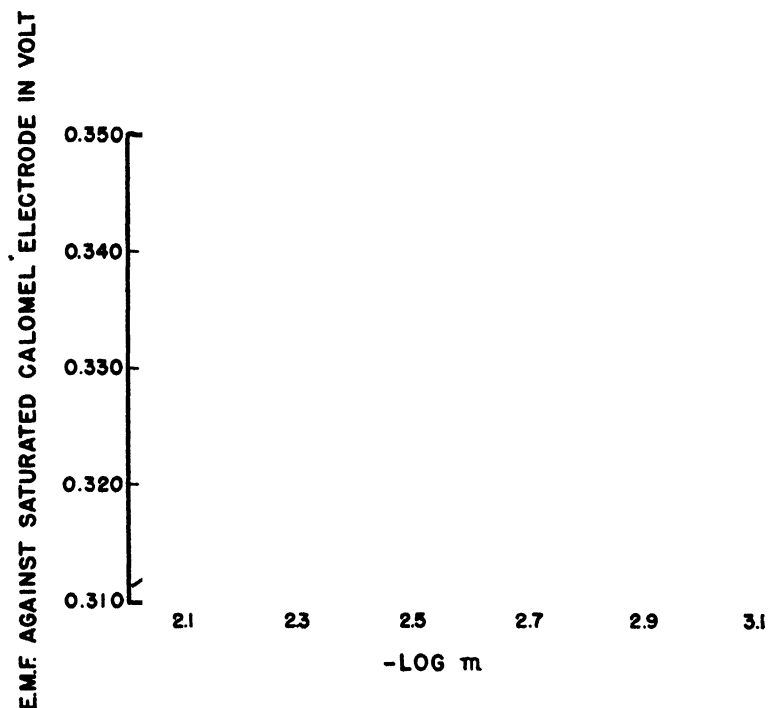


FIG. 6. The variation in potential as a function of total concentration; temperature 30°.

thiourea and 0.005 N in its disulfide. The potentials observed are shown in Fig. 6. It is seen that the logarithmic expression is closely obeyed; the slope is 0.030 as called for by theory. The value of  $E_0$  calculated from this experiment is 0.492.

#### SUMMARY

1. Experimental and theoretical evidence has been presented which indicates that the method of Rykman and Schmidt yields potentials which

are not characteristic of thiol-disulfide systems. Even in the presence of iodide ion, dithiodiglycolic acid has little effect on the potentials of thioglycolic acid solutions.

2. The method used by Ghosh and his coworkers has been shown to give results which are not consistent with their assumed electrode equation. We have obtained data which prove that cysteine potentials at mercury electrodes are due to the cysteine-mercuric cysteinate equilibrium. The potential of this couple at 30° was found to be 0.183 volt. This investigation has shown also that dithizone can extract mercury quantitatively from mercuric cysteinate.

3. Preisler's observations on the thiourea-formamidinium disulfide system have been completely confirmed. The disulfide of ethylene thiourea has been prepared and its properties studied. The reduction potential in 1 N sulfuric acid was found to be 0.495 volt at 30°.

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# STRUCTURAL REQUIREMENTS OF SPECIFIC INHIBITORS FOR $\alpha$ -CHYMOTRYPSIN\*

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Recent studies of the structural requirements of specific substrates for  $\alpha$ -chymotrypsin have led to a relatively narrow definition of the groupings of the substrate which are involved in combination with, and in activation by, this proteolytic enzyme (2-5). These are (1) an aromatic amino acid residue or its structural equivalent, (2) a secondary peptide bond or in its place a group capable of hydrogen bond formation by donation, and (3) the enzymatically susceptible peptide, amide, or ester bond. An attempt has now been made to apply the information obtained in these studies to the search for specific inhibitors for chymotrypsin which are structural analogues of specific substrates. Confidence in the success of such an undertaking was gained from preceding, analogous investigations of carboxypeptidase (6), which have shown that certain structural analogues of substrates which are devoid of the susceptible peptide bond are potent inhibitors for that enzyme. In the work presented here, substrate analogues were also included which contain, in place of the susceptible peptide or ester bond, a bond which is resistant to enzymatic hydrolysis. The negative results previously reported (2) with one of these types of compounds, *i.e.* 1-phenyl-2-acetaminobutanone-3, can now be explained by the unfavorable conditions with respect to solvent composition and enzyme-substrate affinity for the demonstration of inhibitory activity. Under otherwise identical conditions, the degree of competitive inhibition depends on the ratio of the enzyme-substrate dissociation constant,<sup>1</sup>  $K_m$ , to the enzyme-inhibitor dissociation constant,  $K_i$ , inhibition increasing with increasing  $K_m/K_i$  ratios. Accordingly, substrates for chymotrypsin of relatively low, medium, and high  $K_m$  values were employed in this investigation to test the inhibitory activity of the present compounds. Account was also taken of the effect of methanol which in the case of one of the reference substrates, *i.e.* acetyl-L-tyrosin-

\* A preliminary account of this work has been published elsewhere (1).

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<sup>1</sup>  $K_m$  is an explicit function of the rates of reversible combination between enzyme and substrate and of the rate of activation of the enzyme-substrate complex (2):



amide, has been shown to increase  $K_m$  without affecting the rate of activation,  $k'$  (7).

The results of the work presented herein have been integrated with those of preceding studies (2-4) in an attempt to infer certain structural characteristics of the catalytic centers of chymotrypsin.

#### EXPERIMENTAL

*Enzyme*—The preparation of  $\alpha$ -chymotrypsin (IV) was the same as that previously described (2).

*Substrates*—Benzoyl-L-tyrosinamide (BTA), glycyl-L-tyrosinamide (GTA), and acetyl-L-tyrosinamide (ATA) were prepared as previously described (2, 7).

*Inhibitors*—Hydrocinnamic acid (recrystallized before use) and  $\beta$ -phenylethylamine were Eastman products. Benzoyl-L-phenylalanine, benzoyl-D-phenylalanine, benzoyl-o-nitro-L-tyrosine, benzoyl-L-tyrosine, DL- $\alpha$ -benzaminophenylacetic acid,  $\alpha$ -acetaminocinnamic acid, dl-1-phenyl-2-acetaminobutanone-3 (PAAB), and  $\alpha$ -benzylmalonic acid were prepared as previously described (2-4). L-Phenylalanine was a product of Mann Fine Chemicals, and D-phenylalanine was received through the courtesy of Dr. W. H. Stein of The Rockefeller Institute for Medical Research.

N-Acetylglycine (AG) was prepared by the usual acetylation procedure. M.p. found, 208-209°; reported (8), 207-208°.

O,N-Diacetyl-L-tyrosine (DAT) was prepared according to the method of Bergmann and Zervas (9). M.p. found, 168-169°; reported (9), 170°

Calculated, N 5.28; found, N 5.00

dl-1-p-Hydroxyphenyl-2-acetaminobutanone-3 (HPAAB) was prepared according to the directions of Levene and Steiger (10). M.p. found, 116-118°; reported (10), 123-124°.

Calculated, N 6.33; found, N 6.19

*Methods*—Unless otherwise stated, measurements were performed at pH 7.8, 25°. The phosphate buffer concentration was 0.045 M in the presence of 30 per cent methanol and 0.10 M in the absence of methanol. Amidase and esterase activities were determined as previously described (11). Enzyme, substrate, and inhibitor solutions were freshly prepared for each experiment. The preparation of solutions containing the difficultly soluble substrate ATA has already been described (7).

#### Results

The reference substrates used for testing inhibition of chymotryptic activity were glycyl-L-tyrosinamide (GTA), acetyl-L-tyrosinamide (ATA),

and benzoyl-L-tyrosinamide (BTA). Only measurements with BTA as substrate were performed in the presence of 30 per cent methanol. The kinetic constants which characterize the chymotryptic hydrolysis of these substrates are as follows: GTA,  $K_m = 0.118$  and  $k' = 3.9 \times 10^{-3}$ ; BTA (in 30 per cent methanol),  $K_m = 0.042$  and  $k' = 6.5 \times 10^{-3}$ ; ATA,  $K_m = 0.029$  and  $k' = 3.1 \times 10^{-3}$  (2, 4, 12). It has already been shown that at constant initial substrate concentration, the rates of hydrolysis of all three substrates could be approximated by first order reaction equations, at least over the major portion of the rate curves, although first order reaction kinetics do not apply when the initial substrate concentration is varied (2, 4, 13).

For purposes of comparison of the inhibitory activities of the various compounds that have been tested, first order reaction constants have been used, and inhibitory activity was simply expressed as the per cent decrease of the proteolytic coefficient (14) of the reference substrate caused by the addition of the inhibitor, *i.e.*

$$\% \text{ inhibition} = \frac{C - C_i}{C} \times 100 \quad (1)$$

where  $C$  is the proteolytic coefficient without added inhibitor, and  $C_i$  the proteolytic coefficient, at the same initial substrate concentration, in the presence of the inhibitor. Except when otherwise stated, the substrate-inhibitor mole ratio was unity.

In order to establish a common basis for comparing the degree of inhibition obtained from measurements at a single initial substrate concentration with that obtained for two representative inhibitory compounds from more detailed measurements (see below), the following relation was used to calculate enzyme-inhibitor dissociation constants,  $K_i$  (15):

$$\frac{v}{v_i} = \frac{C}{C_i} = 1 + \frac{K_m}{K_i} \cdot \frac{I}{K_m + S} \quad (2)$$

Here,  $v$  and  $v_i$  are the reaction velocities in the absence and presence of inhibitor, respectively, and  $I$  and  $S$  are inhibitor and substrate concentrations, in moles per liter,  $K_m$  and  $K_i$  having already been defined. This equation is predicated on the assumption of competitive inhibition, and as a first approximation, this assumption has been applied to all active, inhibitory compounds of the present investigation.

The results of the present measurements are given in Table I.

Inspection of the data shows that in every case tested the inhibitory activity of a particular compound was higher when tested against ATA or GTA in the absence of methanol than when tested against BTA in the presence of 30 per cent methanol. This result is to be anticipated from the relative magnitudes of the  $K_m$  values of these systems, as well as

TABLE I  
Inhibitory Activities of Structural Analogues of Substrates for Chymotrypsin

Compound tested	Substrate*	Inhibition† per cent	Substrate	Inhibition† per cent	$K_i$ ‡ $10^{-3}M$
1. Benzoyl-DL-phenylalanine	BTA§	1	GTA		2.6
2. Benzoyl-L-phenylalanine			" §	37.6	2.9
3. Benzoyl-D-phenylalanine			" §	54.1	1.5
4. Hydrocinnamic acid	BTA	3	ATA	75.2	0.45
5. Benzoyl-o-nitro-L-tyrosine	"	3¶			
6. Benzoyl-L-tyrosine	"	0-1¶			
7. DL-Phenylalanine	"	0			
8. D-Phenylalanine	ATA	0			
9. L-Phenylalanine	"	0			
10. DL- $\alpha$ -Benzaminophenylacetic acid	BTA	5	ATA	56.8	0.78
11. O,N-Diacetyl-L-tyrosine	"	6	"	34.2	2.0
12. $\alpha$ -Acetaminocinnamic acid	"	13	"	36.7	1.8
13. 1-Phenyl-2-acetaminobutanone-3	"	18	"	56.3	0.79
14. 1-p-Hydroxyphenyl-2-acetaminobutanone-3			"	59.0	0.72
15. $\beta$ -Phenylethylamine			"	18.1**	
16. $\alpha$ -Benzylmalonic acid			"	0	
17. N-Acetylglycine	BTA	0	"	0	

Measurements at pH 7.8, 25°. Unless otherwise stated, the substrate-inhibitor molar ratio was 1:1. The  $K_m$  values for the three substrates are BTA, 0.042; ATA 0.029; GTA, 0.118.

\* The measurements involving BTA as substrate were carried out in the presence of 30 per cent methanol.

† Expressed as per cent change in the proteolytic coefficient, at constant initial substrate concentration, caused by the addition of the inhibitor.

‡ Except when otherwise noted, these values were calculated from kinetic data at a single substrate concentration, with the use of text equation (2), assuming competitive inhibition. In the case of racemic inhibitory compounds, the molar concentration of the racemate was used in these calculations.

§ Substrate-inhibitor molar ratio, 2:1.

|| This value was obtained from the data given in Fig. 1, with the use of text equation (4).

¶ These low values are within the limits of the experimental error. While the insolubility of these compounds in the absence of methanol precluded determinations with GTA or ATA as test substrate, perceptible inhibition may be expected, since the corresponding esters are potent substrates for chymotrypsin (2).

\*\* There is probably considerable error involved in this determination, since under the conditions of the present measurements of amidase activity,  $\beta$ -phenylethylamine was included in the titration, and the blank correction was of a high order.

from the effect of methanol on  $K_m$  (7). It was also found that benzoyl-phenylalanine was a potent inhibitor when tested against GTA, a substrate of relatively low affinity for the enzyme. In contrast to results obtained with carboxypeptidase (6, 13), both optical isomers of the reaction product were inhibitory, although the D isomer was somewhat more so than the L isomer. In agreement with the specificity requirements of specific substrates for chymotrypsin, it was observed that the presence of a free amino group hindered combination with the enzyme, phenylalanine being devoid of inhibitory activity against GTA, in contrast to the inhibitory *N*-benzoylated analogues.<sup>2</sup>

More detailed measurements of enzyme-inhibitor dissociation constants,  $K_i$ , were performed for the anionic inhibitor benzoyl-DL-phenylalanine and for the uncharged inhibitor, PAAB, with use of GTA and ATA, respectively, as substrates. The initial concentration of GTA was varied within the range of 0.0125 to 0.05 M, and that of ATA, within the range of 0.016 to 0.032 M.  $K_m$  and  $K_i$  may be evaluated from such data by means of the equation (16)

$$\frac{1}{v} = \frac{1}{V_{\max}} \left( K_m + \frac{K_m(I)}{K_i} \right) \frac{1}{a} + \frac{1}{V_{\max}} \quad (3)$$

where  $v$  is the initial velocity expressed as moles per liter of substrate hydrolyzed per minute,  $V_{\max}$  the maximum velocity;  $a$  is initial substrate concentration and  $I$  is inhibitor concentration, both in moles per liter. Initial reaction velocities,  $v$ , were more accurately determined by extrapolation, from apparent proteolytic coefficients,  $C = k/e$ , where  $k$  is the first order reaction constant calculated from decimal logarithms and  $e$  is the enzyme concentration in mg. of enzyme N per cc., and  $V_{\max} = k'e$ . Since, for  $e = 1$ ,  $v = -da/dt = 2.3ka = 2.3Ca$ , equation (3) becomes

$$\frac{1}{2.3Ca} = \frac{1}{k'} \left( K_m + \frac{K_m(I)}{K_i} \right) \frac{1}{a} + \frac{1}{k'} \quad (4)$$

where  $k'$  is the specific rate constant for the slowest step between the formation of the enzyme-substrate complex and the subsequent appearance of free enzyme and reaction products (12). In Fig. 1, equation (4) is plotted for the system chymotrypsin-GTA-benzoyl-DL-phenylalanine and for the system chymotrypsin-ATA-PAAB. The common ordinate intercept of the pair of straight lines in each system is indicative of competitive inhibition. The enzyme-inhibitor dissociation constants,  $K_i$ , calculated

<sup>2</sup> The results obtained with benzoyl-L-tyrosine, benzoyl-*o*-nitro-L-tyrosine, and *O,N*-diacetyl-L-tyrosine are, at best, suggestive of inhibitory activity of these compounds (see the foot-note to Table I).

from these data, are  $2.6 \times 10^{-2}$  for benzoyl-DL-phenylalanine and  $7.9 \times 10^{-3}$  for PAAB.

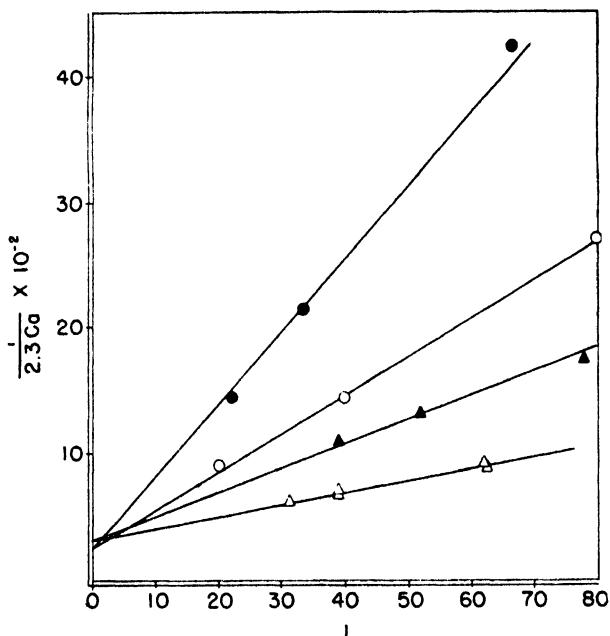
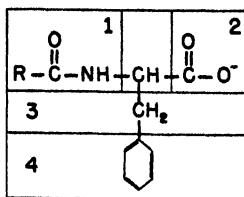


FIG. 1. A plot of equation (4) to demonstrate competitive inhibition of chymotrypsin. The symbols refer to the following systems:  $\Delta$  ATA,  $\blacktriangle$  ATA +  $7.72 \times 10^{-3}$  M PAAB,  $\circ$  GTA,  $\bullet$  GTA +  $2.37 \times 10^{-2}$  M benzoyl-DL-phenylalanine. For calculated values of  $K_i$ , see Table I and the text.

#### DISCUSSION

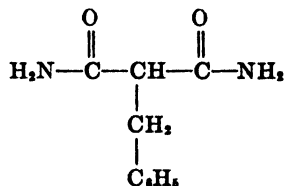
An analysis of the structural characteristics of the present compounds which have been tested for inhibitory activity is facilitated by the accompanying diagrammatic representation of a structural analogue of specific substrates, *e.g.* *N*-acylphenylalanine. This compound fulfils all requirements for specific substrates except that the susceptible peptide (or ester) bond is replaced by a carboxylate group.



It has already been shown that the structural Components 1, 3, and 4 are required for rapid hydrolysis by chymotrypsin (4) and the contribution of all four components to inhibitory activity will now be considered.

*Component 1 (Secondary Peptide Bond)*—While in the case of substrates replacement of this grouping by other electronegative constituents, or its entire omission, greatly decreases the susceptibility of the resulting compound to enzymatic hydrolysis (3, 4), the secondary peptide bond does not seem to be required for enzyme-inhibitor interaction. Thus hydrocinnamic acid<sup>3</sup> is among the most potent inhibitors of the present study.

The relative resistance of ethyl  $\alpha$ -acetaminocinnamate to chymotryptic hydrolysis has been explained by the assumption that only one of several isomeric forms in which this dehydropeptide analogue can exist, fulfils the steric requirements of specific substrates (4). The fact, in contrast, that  $\alpha$ -acetaminocinnamic acid (Compound 12 of Table I) is a relatively active inhibitor, may be taken as further evidence that the steric configuration of the "secondary" peptide bond is inconsequential for interaction of inhibitors with the enzyme.



$\alpha$ -Benzylmalonamide

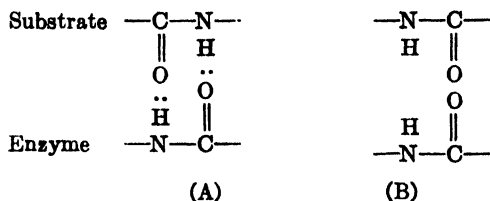
However, if the carbonyl and imino groups of the secondary peptide bond are reversed, as in  $\alpha$ -benzylmalonamide, the resulting compound is entirely inactive as a substrate.<sup>4</sup> In this case, mutual saturation of the hydrogen bonding capacities of the "secondary" amide group and of a complementary peptide group on the enzyme can no longer be achieved, and in addition, close approach to the enzyme surface is hindered by electronic repulsion and steric interference<sup>5</sup> of the two carbonyl oxygens. This is shown in the accompanying Structure B, whereas Structure A shows the favorable conditions for hydrogen bonding of substrates (or inhibitors) with a complementary peptide group on the enzyme surface. A similar type of bonding provides at least partial binding of the susceptible peptide or ester group of substrates, or of carboxylate or ketone

<sup>3</sup> It is of interest to note that hydrocinnamic acid fulfils also the structural requirements for inhibitors for another proteolytic enzyme, *i.e.*, carboxypeptidase (6).

<sup>4</sup> Unpublished experiments by the authors.

<sup>5</sup> Manipulations with Fisher-Hirschfelder models support this conclusion.

groups of inhibitors to an adjacent peptide group on the enzyme. These considerations apply equally to *d* isomers of specific substrates, since in these compounds the positions of the secondary peptide bond and of the susceptible bond will be interchanged with respect to the peptide groups of the catalytic centers, thus creating conditions analogous to those just described for  $\alpha$ -benzylmalonamide.<sup>6</sup>



If a carboxylate group is introduced in place of the secondary peptide group ( $\alpha$ -benzylmalonic acid), the inhibitory power is likewise lost.

The presence in specific substrates of a positive charge in place of the secondary peptide group (i.e. tyrosinamide or tyrosine ethyl ester (4, 11)), greatly decreases the susceptibility of the resulting compound to chymotryptic hydrolysis. A similar effect exists for inhibitory compounds, as evidenced by the failure of either isomeric form of phenylalanine to cause any measurable inhibition. However, the presence of an  $\alpha$ -amino group in compounds which are devoid of the secondary  $\alpha$ -carboxylate group ( $\beta$ -phenylethylamine) does not destroy inhibitory activity.

**Component 2 (Carboxylate Group)**—While compounds containing a carboxylate group in the  $\alpha$  position are inhibitory, provided the remaining structural requirements are fulfilled, this group is not essential for inhibitory activity. One of the most potent inhibitors for chymotrypsin as yet found results when the susceptible bond is replaced by a methyl ketone group which is resistant to enzymatic hydrolysis. This is demonstrated by results obtained with PAAB (Compound 13 of Table I) and HPAAB (Compound 14 of Table I) which are the structural analogues of the specific substrates *N*-acylphenylalanine methyl ester and *N*-acyltyrosine ethyl ester (2-4). Replacement of the carboxylate group by an amino group likewise yields an inhibitory compound (see above).

**Component 3 (Methylene Group)**—When this grouping is eliminated from a typical substrate (benzoylphenylalanine ethyl ester), the resulting compound (ethyl *DL*- $\alpha$ -benzaminophenylacetate) is resistant to chymo-

<sup>6</sup> This factor differentiates *d* isomers of substrates which contain a secondary peptide bond from those which are devoid of this bond. Thus both isomers of methyl *dl*- $\alpha$ -chlorophenylacetate are hydrolyzed at the same slow rate, whereas in the case of methyl phenyllactate, the *l* isomer, which offers some measure of interaction through the hydroxyl group, is more rapidly hydrolyzed than the *d* isomer (3).

tryptic hydrolysis. In contrast, the corresponding acid, *i.e.* DL- $\alpha$ -benzaminophenylacetic acid (Compound 10 of Table I) has a relatively high inhibitory activity. This result is in contrast to analogous studies of specific inhibitors for carboxypeptidase (6) which have shown that the distance of separation of the phenyl group from the  $\alpha$ -carbon atom is of critical importance.

*Component 3 (Phenyl Ring)*—It has been shown that the phenyl ring, or its electronic equivalent, is an essential grouping in specific substrates (4). The lack of inhibition observed with acetylglycine indicates the necessity of this structural component for specific inhibitors as well.

Consideration of the present experimental findings, in conjunction with those of the preceding analysis of the structural requirements of specific substrates for chymotrypsin (2-4), leads to certain generalizations which may be stated as follows: (1) The structural requirements for specific inhibitors are less stringent than those for specific substrates. Thus benzoyl-D-phenylalanine, hydrocinnamic acid,  $\alpha$ -acetaminocinnamic acid, and DL- $\alpha$ -benzaminophenylacetic acid are active inhibitors, whereas the corresponding esters are only slowly hydrolyzed by chymotrypsin, if at all (3, 4). The reverse situation, *i.e.* a substrate with less stringent requirements than the corresponding acid, has not yet been encountered. (2) Both ionic and neutral molecules can serve as inhibitors. (3) Neither of the two compounds which bear two charges adjacent to the  $\alpha$ -carbon atom, *i.e.* phenylalanine and  $\alpha$ -benzylmalonic acid, exhibits inhibitory activity. (4) Whereas only the L isomer of specific substrates is hydrolyzed, both optical isomers of the corresponding inhibitory analogues are specific inhibitors (*i.e.* benzoyl-DL-phenylalanine). (5) The secondary peptide group is not requisite for inhibitors if a negatively (carboxylate) or positively (amino) charged group is adjacent to the  $\alpha$ -carbon atom.

The foregoing considerations converge toward the view that the active centers of chymotrypsin possess a complementary configuration to that of specific substrates and inhibitors, part of such a complementary configuration being two adjacent peptide groups along a peptide chain. Additional complementary structures have to exist to account for the side chain specificity of substrates and inhibitors, which decreases in the order of tyrosine, phenylalanine, tryptophan, methionine (4). Although it has been shown that chemical modification of the total tyrosine residues of chymotrypsin causes enzyme inactivation (17), whereas fractional oxidation by tyrosinase (18-20) does not, the evidence is insufficient to assign to a critically placed tyrosine residue in chymotrypsin a catalytic function.<sup>7</sup> A more detailed discussion of this aspect of the problem of the

<sup>7</sup> It is of interest to note that, on the basis of the amino acid analyses of Brand (quoted in Northrop *et al.* (21)),  $\alpha$ -chymotrypsin contains 3.1 tyrosine residues per



enzymatic specificity of chymotrypsin and of other proteolytic enzymes will be presented elsewhere.<sup>8</sup>

It is to be emphasized that these conclusions are predicated on the assumption that the active inhibitors which have been studied interfere with the combination of the substrates with the catalytically active centers on the enzyme surface. Combination of substrates, and inhibitors, respectively, with the same groupings on the enzyme has not been definitely established. However, for two specific compounds, *i.e.* benzoyl-DL-phenylalanine and PAAB, competitive inhibition has been demonstrated, and the structural resemblance of all inhibitory compounds to specific substrates provides presumptive evidence that in every case the enzymatically active groupings or closely adjacent regions on the enzyme surface are involved.

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#### SUMMARY

A series of structural analogues of specific substrates of chymotrypsin has been prepared and tested for inhibitory activity. It has been found that analogues which are either devoid of the susceptible bond or which contain in its place a (ketone) group which is resistant to chymotryptic hydrolysis can serve as inhibitors provided other structural requirements of substrates are fulfilled.

The structural requirements for specific inhibitors are less stringent than those for specific substrates.

The secondary peptide group has been found to be dispensible for inhibitors. Among the most active specific inhibitors are benzoyl-DL-phenylalanine and *dl*-1-phenyl-2-acetaminobutanone-3, both of these compounds exhibiting competitive inhibition.

Considerations of specificity requirements of specific substrates and inhibitors have led to a tentative formulation of the configuration of the catalytic center of chymotrypsin.

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20,000 molecular weight unit (22). By means of the same source of data for chymotrypsinogen, a phenylalanine content of 4.3 residues per 20,000 molecular weight unit is calculated. In the light of recent studies of the stoichiometric relations of the inhibition of chymotrypsin by diisopropyl fluorophosphate (23) it seems unlikely that more than one catalytic center exists per chymotrypsin molecule.

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# THE MICROBIOLOGICAL ASSAY OF VITAMIN B<sub>12</sub> WITH LACTOBACILLUS LEICHMANNII

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The organism *Lactobacillus lactis* Dorner (ATCC 8000) was reported to need two unidentified factors for growth (1), one of which (LLD factor) was present in liver extracts in amounts which bore a close relationship to the potency of the extracts in producing a remission of pernicious anemia. Later investigations (2) led to the separation from liver of a red crystalline compound, "vitamin B<sub>12</sub>," which was therapeutically effective in pernicious anemia and which was found to be highly active for *L. lactis* Dorner (3), showing vitamin B<sub>12</sub> to be "either wholly or partially responsible for the LLD growth activity observed for liver extracts." Other investigations (4) demonstrated that thymidine could function as a source of LLD factor and that thymidine and other desoxyribosides were essential growth factors for various lactic acid bacteria (5-7).

The attempted use of *L. lactis* Dorner as an assay organism for vitamin B<sub>12</sub> has led to difficulty in obtaining reproducible results (8) and Shorb and Briggs (9) have shown that under certain conditions this organism dissociates into a form which does not require vitamin B<sub>12</sub>.

It was found that *Lactobacillus leichmannii* 313 (ATCC 7830) would respond to crystalline antipernicious anemia factor on a chemically defined culture medium (10) and the use of this organism for assay purposes was suggested. A second strain of *L. leichmannii* (ATCC 4797) was simultaneously reported to respond to concentrated preparations of a growth factor for mice, termed the animal protein factor, which was indicated as being closely related to vitamin B<sub>12</sub> (8). Following these publications, both these strains of *L. leichmannii* have been used for the assay of vitamin B<sub>12</sub> (11, 12).

The present communication deals with a study of the factors influencing the response of these organisms to vitamin B<sub>12</sub>. It was found that the addition of a reducing agent such as thioglycolic acid stimulates the growth of the organisms and protects vitamin B<sub>12</sub> from destruction during the autoclaving of the samples. This finding was noted in a preliminary communication (13).

## EXPERIMENTAL

*Assay Organisms and Basal Medium*—*L. leichmannii* 313 (ATCC 7830) and *L. leichmannii* ATCC 4797 were used as assay organisms. The inoculum was prepared as follows: The culture was maintained on a medium containing liquid skim milk 1000 ml., tomato juice (not clarified) 100 ml., and yeast extract 5 gm., with a final pH of 6.5. Transfers from a 24 hour milk-medium culture were made into 10 ml. portions of the basal medium

TABLE I  
*Composition of 500 Ml. of Double Strength Basal Medium\**

	gm.		mg.
Glucose	10.0	Adenine	10.0
Sodium acetate, anhydrous	10.0	Guanine	10.0
"    citrate	10.0	Uracil	10.0
Trypsin-digested casein†	1.2	<i>p</i> -Aminobenzoic acid	2.5
Acid-hydrolyzed " ‡	5.0	Nicotinic acid	1.0
K <sub>2</sub> HPO <sub>4</sub>	3.0	Riboflavin	0.8
KH <sub>2</sub> PO <sub>4</sub>	3.0	Pyridoxal	0.4
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2.8	Pyridoxine	0.2
MnSO <sub>4</sub> ·4H <sub>2</sub> O	0.6	Calcium pantothenate	0.2
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.17	Thiamine	0.2
Tween 80	1.0	Biotin	0.002
L-Cystine	0.2	Folic acid	0.002
Asparagine	0.1		
DL-Tryptophan	0.1		

\* Thioglycolic acid, 0.2 gm., is included when the medium is used for assay purposes. It is added just before autoclaving.

† 100 gm. of Labco vitamin-free casein were added to 1000 ml. of 0.8 per cent sodium bicarbonate and steamed 5 to 10 minutes. The solution was adjusted to pH 8, 2 gm. of trypsin or pancreatin were added, and the mixture was incubated 48 hours at 37° (pH readjusted to 8 after 12 to 24 hours). The digest was neutralized, filtered, brought to pH 3, adsorbed twice with 15 gm. portions of norit 231, and the volume adjusted to 1 liter (equivalent to 100 mg. of casein per ml.). If blanks in the assay were too high, a third norit adsorption was made.

‡ 900 gm. of Labco vitamin-free casein were refluxed with 9000 ml. of 6 N hydrochloric acid for 9 hours. The hydrolyzed mixture was concentrated to a thick syrup, diluted to 4.5 liters, neutralized to pH 3.0, and filtered. The solution was decolorized by two adsorptions with 90 gm. portions of Darco G-60 or norit.

(without thioglycolic acid) containing 2.5 ml. of refined liver extract, 10 U. S. P. units per ml. per liter of single strength medium. This tube was incubated for 24 hours; the cells were then centrifuged and resuspended in 60 ml. of saline. 1 drop of this inoculum was added in each assay tube.

The composition of the basal medium is shown in Table I. The assays were carried out in 12 × 100 mm. Pyrex test-tubes in a total volume of 2.0 ml. After sterilization of the sample *plus* medium for 15 minutes the

tubes were inoculated and incubated at 37° for varying lengths of time. After incubation, turbidities were measured in a Coleman spectrophotometer at 600 m $\mu$ .

**Assay Standards**—Crystalline antipernicious anemia factor<sup>1</sup> (14) or vitamin B<sub>12</sub> (Cobione, Merck) was used for obtaining standard response curves. These materials gave identical responses; a suspected difference in their phosphorus content (15) has recently been shown not to exist and the preparations are considered identical (16). The term "liver extract" refers to concentrated liver extract, 15 units per ml. (Lederle Laboratories).

**Results**—In preliminary experiments, two apparently conflicting phenomena were noted in relation to autoclaving. In spite of the known thermostability of vitamin B<sub>12</sub> (17), it was found that the response obtained in certain samples was increased when the period of autoclaving the assay tubes was prolonged. In contrast, almost no growth was obtained when the medium was sterilized by steaming, even though large amounts of vitamin B<sub>12</sub> were added. This suggested that an essential growth factor was formed when the medium containing glucose was subjected to autoclaving and that this growth essential was not formed when the medium was sterilized by the less drastic heat treatment involved in steaming. Certain decomposition products derived from sugars by autoclaving with the basal medium have been noted to be required for "prompt growth" of *Lactobacillus bulgaricus* (18).

In order to investigate this point the medium was autoclaved and the samples were sterile-filtered through a Seitz filter, and then diluted in sterile water and added aseptically to the cooled medium. A greater response was obtained than when the samples were added to the medium before autoclaving. From these results, illustrated in Fig. 1, it appears that vitamin B<sub>12</sub> was partially destroyed by autoclaving and that a second growth factor was produced in the medium during the autoclaving process.

In the next series of experiments the effect of thioglycolic acid was investigated. When this substance was included in the medium, similar results were obtained when the samples were added before autoclaving and when the samples were added aseptically to autoclaved medium containing thioglycolic acid. These results, which are also illustrated in Fig. 1, indicate that thioglycolic acid protected vitamin B<sub>12</sub> during the autoclaving process which was used in the assay.

The next point investigated was the effect of different compounds in replacing the growth factor which was formed in the medium during autoclaving. When glucose was autoclaved separately from the remainder of the medium and vitamin B<sub>12</sub> was added aseptically, almost no growth was

<sup>1</sup> Crystalline antipernicious anemia factor was kindly furnished by the Glaxo Laboratories, Ltd.

obtained, even with large amounts of vitamin B<sub>12</sub>. If sucrose was used instead of glucose, no growth was obtained even if the sucrose was autoclaved with the medium for 20 minutes. However, when thioglycolic acid was added, a growth response was obtained to vitamin B<sub>12</sub> both in the medium containing glucose which had been autoclaved separately and in the sucrose medium. The addition of methylglyoxal produced similar results with the first of these two media. These results, which are summarized in Table II, indicate that the failure to obtain growth was due to the fact

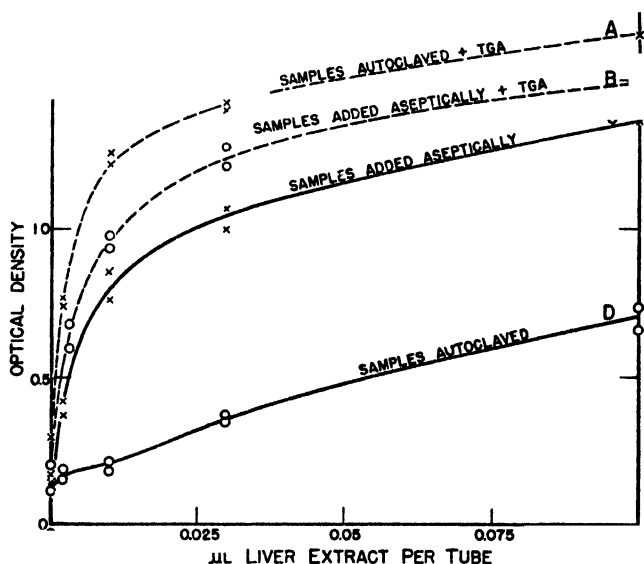


FIG. 1. Effect of thioglycolic acid and of aseptic addition of samples on the response of *L. leichmannii* 313 to liver extract. Curve A, samples autoclaved with medium containing 0.2 mg. of thioglycolic acid (TGA) per tube; Curve B, sterile samples added aseptically to autoclaved medium containing 0.2 mg. of TGA per tube; Curve C, samples added aseptically to autoclaved medium; Curve D, sample autoclaved with medium, no thioglycolic acid added.

that glucose did not form a reducing agent unless it was autoclaved in contact with the rest of the medium and that sucrose did not form a reducing agent even when autoclaved together with the medium. Further results presented in Table II indicated that ascorbic acid and cysteine could replace thioglycolic acid as reducing agents.

In the next series of experiments, the effects of thioglycolic acid on the growth response to liver extract and to crystalline antipernicious anemia factor were compared. The growth response to liver extract was markedly increased by adding thioglycolic acid. This increase was much more

marked than that obtained by adding thioglycolic acid to crystalline anti-pernicious anemia factor (Fig. 2). The vitamin B<sub>12</sub> content of liver measured by comparison with the crystalline factor was apparently increased by the addition of thioglycolic acid to the medium. Two explanations may be offered for the difference between liver extract and crystalline anti-pernicious anemia factor with respect to the effect produced by adding thioglycolic acid. (1) There may be a microbiologically inactive form of vitamin B<sub>12</sub> in liver extract which is activated when it is autoclaved with thioglycolic acid. (2) Vitamin B<sub>12</sub> may be more labile in liver extract than in pure solution when autoclaved in the absence of thioglycolic acid.

TABLE II

*Effect of Methods of Sterilization and Type of Carbohydrate on Growth of L. leichmannii 515 (ATCC 7830)*

Carbohydrate used	Treatment	Reducing agent added per ml. medium	Growth (optical density) obtained with various levels of vitamin B <sub>12</sub>		
			μg/m.	μg/m.	μg/m.
Glucose . . . . .	*	None	None	0.1	1.0
" . . . . .	*	0.2 mg. TGA†	0.09	0.15	0.79
" . . . . .	*	0.2 " cysteine	0.40	0.88	1.38
" . . . . .	*	0.5 " ascorbic acid	0.31	0.55	1.35
" . . . . .	†	None	0.19	0.61	1.4
" . . . . .	†	0.2 mg. TGA	0.02	0.01	0.14
" . . . . .	†	0.2 " methylglyoxal	0.26	0.47	0.81
Sucrose . . . . .	*	None	0.07	0.25	0.73
" . . . . .	*	0.2 mg. TGA	0.02	0.05	0.28
" . . . . .	*	0.2 mg. TGA	0.35	0.68	1.0

\* Carbohydrate at 1 per cent level autoclaved with medium.

† TGA = thioglycolic acid.

‡ Carbohydrate autoclaved separately and added aseptically to autoclaved medium after cooling.

These points were explored by adding sterile-filtered liver extract and crystalline antipernicious anemia factor aseptically to the medium after the latter had been autoclaved. It was found that the responses obtained by aseptic addition were the same as those obtained when the materials were autoclaved in the presence of thioglycolic acid. This observation indicates the absence in liver extract of any appreciable quantity of a "microbiologically inactive form" of vitamin B<sub>12</sub> which was activated by thioglycolic acid. These results are illustrated in Fig. 2 and in Table III.

In the next series of experiments a study was made of the conditions influencing the stability of vitamin B<sub>12</sub> in liver extract and in pure solution. It was found that the addition of the basal medium to the liver extract



accelerated the decrease in potency. The addition of thioglycolic acid to liver extract diluted with water did not completely protect the vitamin

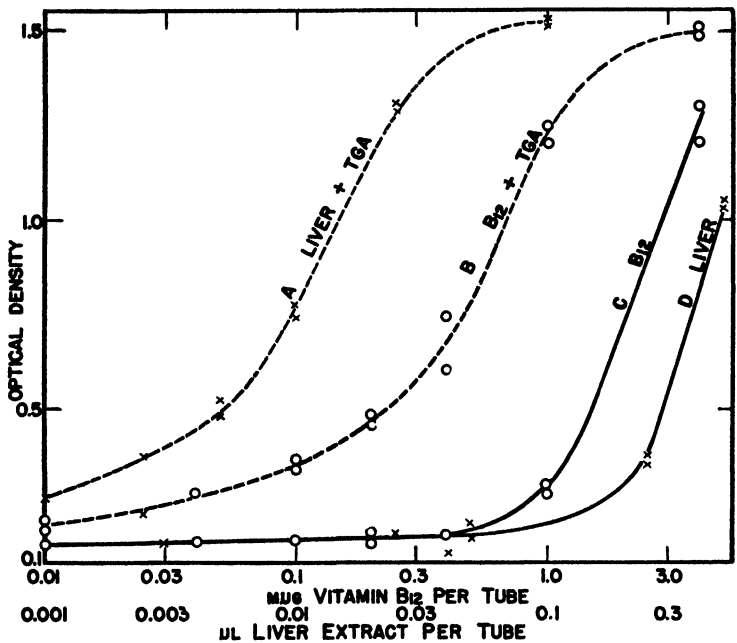


FIG. 2. Effect of thioglycolic acid (TGA) on the response of *L. leichmannii* 313 to liver extract (Curves A and D) and vitamin B<sub>12</sub> (Curves B and C). Curves A and B, 0.2 mg. of thioglycolic acid per ml. of medium; Curves C and D, medium without thioglycolic acid.

TABLE III  
Effect of Thioglycolic Acid (TGA) on Destruction of Vitamin B<sub>12</sub> in Liver Extract

Treatment of liver extract	Loss of vitamin B <sub>12</sub> activity
	per cent
None.....	0
Diluted 1:100 in water; autoclaved 10 min.....	80
“ 1:100 “ “ + 0.20 mg. TGA per ml. at pH 6.5; autoclaved 10 min.....	65
Diluted 1:100 in basal medium; autoclaved 10 min.....	92
“ 1:100 “ “ “ + 0.20 mg. TGA per ml. at pH 6.5; autoclaved 10 min.....	20

B<sub>12</sub> as indicated by microbiological activity of the samples, but the addition of thioglycolic acid to liver extract diluted with the basal medium gave

good protection. Thus thioglycolic acid exerted its maximum stabilizing effect in the presence of the basal medium.

*Use of Asparagus Concentrate As Source of Stabilizing and Reducing Agent*—A concentrate was prepared from asparagus as follows: 200 ml. (100 gm. of solids) of asparagus juice concentrate<sup>2</sup> were diluted to 1 liter and adjusted to approximately pH 7.5 with 60 gm. of sodium bicarbonate. The solution was filtered and then extracted continuously with normal butanol *in vacuo* for 72 hours, following which the butanol was removed by evaporation under reduced pressure in an atmosphere of nitrogen. The volume of the final solution was adjusted to 1 liter and 10 ml. were used in 500 ml. of double strength medium. It was found that this concentrate could be used to replace the enzyme-digested casein in the medium in Table I and that it also had a protective effect on the potency of the samples

TABLE IV

. *Effect of Various Supplements on Microbiological Assay of Vitamin B<sub>12</sub>*

Organism, *L. leichmannii* 313 (ATCC 7830); sterilization time, 10 minutes at 120°. The results are expressed in micrograms of vitamin B<sub>12</sub> found per ml.

Liver extract	Medium, no addition	Medium + 0.02 per cent thioglycolic acid	Medium + 5 per cent asparagus concentrate	Medium autoclaved alone; sample added aseptically
1	2.1	30, 35	27.	33
2		30		
3		28		
4		17	20	27
5	3, 2.0	30, 27	31	32, 27
6	6, 2.1	40, 41		

during autoclaving similar to that exerted by thioglycolic acid. The basal medium in which the enzyme-digested casein was replaced by an asparagus concentrate at a level of 5 ml. per liter produced a lower response (optical density 0.2) than that produced by the basal medium containing enzyme-digested casein and thioglycolic acid (optical density 0.5). The thioglycolic acid-like effect of the asparagus concentrate was found to be variable, and in some cases more consistent results were obtained by adding both the asparagus concentrate and thioglycolic acid. The assay values obtained for liver extract or vitamin B<sub>12</sub> solutions by using the medium containing enzyme-digested casein and thioglycolic acid were not substantially different from those obtained with the modified medium containing asparagus concentrate. This is illustrated in Table IV.

<sup>2</sup> Asparagus concentrate was kindly supplied by the Western Regional Research Laboratory, United States Department of Agriculture, Albany 4, California

*Correction for Effect of Desoxyribosides*—It has been demonstrated that growth of *L. leichmannii* is obtained when thymidine or other desoxyribosides are used to replace vitamin B<sub>12</sub> (5–8, 10) and hence it is necessary to devise a method for separating vitamin B<sub>12</sub> from the desoxyribosides if *L. leichmannii* is to be used for assaying the vitamin B<sub>12</sub> content of natural materials. It has been shown that the antipernicious anemia potency of liver fractions (19) and vitamin B<sub>12</sub> (17) in pure solutions is destroyed by treatment with sodium hydroxide. In the present investigation it was found that vitamin B<sub>12</sub> was destroyed by heating with 0.2 N NaOH at 100° for 30 minutes and that under these conditions the desoxyribosides of thymine, guanine, and hypoxanthine were not affected. Liver extract was assayed before and after this treatment with alkali, and the difference between the two assay values was used to express the vitamin B<sub>12</sub> content. Usually about 97 per cent of the total microbiological activity of liver extract was found to disappear upon treatment with alkali, indicating that desoxyribosides were responsible for only a small fraction of the microbiological activity of the extracts.

*Use of L. leichmannii ATCC 4797* — This organism was also used for assay purposes. It required 48 hours to reach nearly maximum growth, compared with 20 hours for the more rapidly growing strain 313.

*Comparison of Results Obtained with Different Microbiological Procedures and with Chick Assays* — Assays of a number of liver extracts were made with the different microbiological procedures described here and the results are presented in Table IV. The data show that the results obtained by aseptic addition of the sterile sample were essentially the same as those obtained by the use of thioglycolic acid or asparagus concentrate. These values in turn agreed well with the results obtained by chick assay against a crystalline vitamin B<sub>12</sub> standard.<sup>a</sup>

*Modifications of Procedure for Use in Routine Assays* — Sodium citrate and enzyme-digested casein are omitted from the medium described in Table I when routine assays are to be made, and 10 ml. of the asparagus juice fraction, described above, are included in 500 ml. of double strength medium. The salts are replaced by K<sub>2</sub>HPO<sub>4</sub> 1 gm., KH<sub>2</sub>PO<sub>4</sub> 1 gm. (Salt Mixture A); MgSO<sub>4</sub>·7H<sub>2</sub>O 0.4 gm., NaCl 20 mg., FeSO<sub>4</sub>·4H<sub>2</sub>O 20 mg., MnSO<sub>4</sub>·4H<sub>2</sub>O 20 mg. (Salt Mixture B). The medium is adjusted to pH 6.8 to 6.9, as determined electrometrically, heated until the precipitate flocculates, and filtered. Thioglycolic acid is added just prior to sterilization and the pH is readjusted to 6.8 to 6.9. The assay is carried out in 18 × 150 mm. Pyrex culture tubes with a total volume of 10 ml. of medium. After autoclaving for 10 minutes, the tubes are inoculated and incubated at 37° for 40 hours, following which the turbidity is measured in a photo-

<sup>a</sup> Manuscript in preparation.

electric colorimeter. The content is calculated by reference to a response curve made with crystalline vitamin B<sub>12</sub> over a range of 0.05 to 1.0 m $\mu$ gm.

The modifications in the preceding paragraph represent changes in the assay procedure which were developed as a result of the present investigation. The medium described in Table I should be modified in accordance with these recommendations when assays are to be made.

#### DISCUSSION

The relation of the vitamin B<sub>12</sub> content of liver extracts, as determined by microbiological assay, to clinical potency, as measured by tests in patients with pernicious anemia, is of much interest. Purine and pyrimidine desoxyribosides will produce a response in the assay with *L. leichmannii*, although *Euglena gracilis* has been shown not to respond to thymidine (20). In the present investigation it was found that a correction could be made for the desoxyriboside content of liver extracts by subtracting the alkali-stable potency from the potency of the untreated extract. The alkali-stable potency represents the desoxyribosides. These have been shown to have about 0.03 per cent of the potency of vitamin B<sub>12</sub> for *L. leichmannii* (6, 10). Thymidine has not been found to have activity in pernicious anemia (21). The presence of growth factors in liver extract other than vitamin B<sub>12</sub> and desoxyribosides is of interest both from the standpoint of their possible clinical effects and of their activity in microbiological assays. It has been shown (22) that antipernicious anemia liver extracts may be good sources of a growth factor for *Leuconostoc citrovorum* 8081 which has been differentiated from the factor for *L. leichmannii* in such extracts, presumably vitamin B<sub>12</sub>, by migration in an electrolytic apparatus (23). Vitamin B<sub>12</sub> will not replace the *citrovorum* factor in promoting the growth of *L. citrovorum* nor does the *citrovorum* factor appear to promote the growth of *L. leichmannii* (24).

The possible presence of "conjugates" of vitamin B<sub>12</sub>, incompletely available microbiologically, in liver extracts remains to be explored. Certain fermented materials have shown potencies for *L. leichmannii* which corresponded to only a fraction of their potencies in the chick assay for animal protein factor (25). This may indicate the possible existence of such conjugates in nature.

#### SUMMARY

1. The use of two strains of *Lactobacillus leichmannii* for the assay of vitamin B<sub>12</sub> in liver extracts is described. These are designated respectively strains 313 (ATCC 7830) and ATCC 4797. Both strains gave satisfactory results; the former grows more rapidly, enabling the use of a short assay period.

2. The addition of a reducing agent was necessary to protect vitamin B<sub>12</sub> from destruction when the assay tubes were autoclaved. Alternatively, destruction of vitamin B<sub>12</sub> could be prevented by the addition of the sterile assay samples to the autoclaved medium.

3. A growth factor was produced in the medium during autoclaving, but, when sucrose was substituted for glucose in the medium, the factor was not produced. The growth factor was not needed if thioglycolic acid or an extract of asparagus was included in the medium.

4. The effect of desoxyribosides, which replace vitamin B<sub>12</sub> for the growth of *L. leichmannii*, was measured and corrected by assaying the samples before and after alkaline hydrolysis which destroyed vitamin B<sub>12</sub>.

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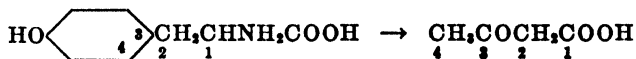
# KETONE BODY FORMATION FROM TYROSINE\*

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In a preliminary communication (1) we reported results of a study with isotopic tyrosine which, as illustrated, indicated that in the metabolic conversion of tyrosine to acetoacetate the carboxyl and  $\alpha$ -carbons of the latter are derived respectively from the  $\alpha$ - and  $\beta$ -carbons of tyrosine, the  $\beta$ - and  $\gamma$ -carbons being provided by 2 tyrosine ring carbons. The present report is concerned with a more detailed account of this investigation.



## EXPERIMENTAL

The experimental procedure consisted of incubation of liver slices of the rat with isotopic tyrosine, labeled with  $\text{C}^{14}$  in the  $\beta$ -carbon, followed by determination of the distribution of the  $\text{C}^{14}$  among the 4 carbons of the acetoacetate which is formed metabolically under these conditions. The starting material was DL-tyrosine,  $\beta$ -labeled with  $\text{C}^{14}$ , obtained through the kindness of Dr. Melvin Calvin of the Radiation Laboratory, University of California. This substance, in an amount of 2.34 mg., was added to 230 mg. of L-tyrosine and twice crystallized from water, yielding 179 mg. of essentially pure L-tyrosine. For assay, a sample was oxidized to  $\text{CO}_2$  (2), which was isolated as barium carbonate, spread in an "infinitely thick" layer 5 sq. cm. in area and counted under a thin window counter (3). The activity was 678 counts per minute. All samples derived from these experiments were assayed similarly; their specific activities are reported as relative values based on an assumed value of 100 for the L-tyrosine.

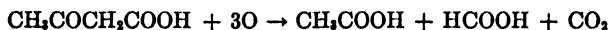
The experiments were made in Warburg-type flasks of large capacity. Procedures for incubation, recovery of respiratory  $\text{CO}_2$ , and thermal decarboxylation of acetoacetate were essentially the same as described previously (4). The labeled tyrosine was added to the flasks as an approximately saturated solution neutralized to pH 7.4. The liver slices, from adult male rats fasted 24 hours, were prepared with the Stadie-Riggs slicer (5).

*Distribution of  $\text{C}^{14}$  in Acetoacetate*—In the first experiment 33.5 mg. of the labeled tyrosine were incubated 2 hours at  $37^\circ$  in oxygen with 4 gm.

\* This work was supported by the Sun Oil Company, Philadelphia, and aided by a grant from the National Cancer Institute, United States Public Health Service.

of liver slices. The acetone-mercury complex, obtained by thermal decarboxylation of the metabolically formed acetoacetate, had an activity of 138 counts per minute, whereas the  $\text{CO}_2$ , representing the acetoacetate carboxyl, had an activity of only 5 counts per minute. The acetone-mercury complex was dissolved in  $\text{HCl}$ , and the acetone distilled into an ice-cold alkaline hypoiodite solution. The precipitated iodoform, collected by extraction with chloroform, had an activity of 51 counts per minute. This preliminary experiment thus established the presence of radioactivity in the  $\alpha$ - or  $\gamma$ -carbon of acetoacetate, or both, and left open the question of the presence of radioactivity in the  $\beta$  position.

It was evident that further information on the distribution of  $\text{C}^{14}$  would require a supplementary degradation procedure, necessarily one which would distinguish between the  $\alpha$  and  $\gamma$  positions. It seemed probable that a mild oxidation of acetoacetate would yield acetic acid from the  $\beta$ - and  $\gamma$ -carbons. In agreement with this expectation a very simple degradation procedure was developed, based on oxidation with permanganate in acid solution at low temperature, yielding acetic, formic, and carbonic acids in accordance with the following equation:



Under the experimental conditions employed not all of the  $\alpha$ -carbon is recovered as formate, but some is oxidized to  $\text{CO}_2$ , resulting in formation of the three products in an approximate molar ratio of 1:0.4:1.6. In a typical experiment 1.00 mm of acetoacetate yielded 1.02 mm of acetate, 0.44 mm of formate, and 1.50 mm of  $\text{CO}_2$ . The detailed procedure is given in the next section.

*Oxidation of Acetoacetate with Permanganate*—For convenience and completeness in the recovery of the volatile acids the amounts of acetoacetate taken ranged between 0.5 and 1.0 mm. When smaller amounts were available, as in these experiments, sufficient carrier was added to give a total of around 0.5 mm. Smaller quantities could undoubtedly be degraded by appropriate microprocedures.

The solution of acetoacetate (copper-lime filtrates in these experiments) is washed into a 200 ml. three-necked flask carrying a dropping funnel, a lead-in tube extending to the bottom, and a condenser, the top of which has a tube leading to an absorption tower. The apparatus used is essentially the same as the one described previously for persulfate oxidation (2). The flask is immersed in an ice bath, and sufficient strong sulfuric acid and water added to give 100 ml. of a 1 M acid solution.  $\text{CO}_2$ -free air is drawn rapidly through the flask for about 5 minutes to remove any dissolved  $\text{CO}_2$ ; then 10 ml. of an 0.5 M  $\text{CO}_2$ -free  $\text{NaOH}$  solution are placed in the bead tower and, with a moderate stream of air passing through the

solution and into the bead tower, a sufficient quantity of 1.5 N  $\text{KMnO}_4$  solution is added to the acetoacetate to give a permanent pink color (8 to 10 ml. were required in these experiments). About 30 minutes are allowed for complete absorption of the  $\text{CO}_2$ , after which the alkaline solution in the bead tower is washed back into the flask and the carbonate precipitated by the addition of barium chloride.

To recover the volatile acids the excess permanganate is destroyed by dropwise addition of 3 per cent hydrogen peroxide, any excess peroxide is decomposed by adding a few drops of very dilute permanganate to a permanent pink color, and the solution is steam-distilled until no more acid comes over, each portion of 50 ml. being titrated with standard alkali. The neutralized solution is evaporated to 50 ml., cooled, and acidified with 2 ml. of 18 N sulfuric acid. This is placed on the same set-up used for the original oxidation, 10 ml. of 10 per cent mercuric sulfate solution are added, and with a moderate stream of air passing through, the solution is refluxed half an hour. The  $\text{CO}_2$  resulting from the oxidation of the formate is again collected in a bead tower and precipitated and collected as barium carbonate.

The acetate remaining is recovered and determined by distillation essentially according to the method of Friedemann (6). Duclaux curves on this fraction, obtained from synthetic or metabolic acetoacetate, are indistinguishable from those of pure acetic acid.

*Oxidation of Synthetic, Labeled Acetoacetate*—From the products and their yields in this process it seemed quite certain that the acetate, formate, and  $\text{CO}_2$  were derived respectively from the  $\beta$  and  $\gamma$ , the  $\alpha$ , and the carboxyl carbons. To check this point, however, synthetic  $\beta$ - and  $\text{COOH}$ -labeled acetoacetate,  $\text{CH}_3\text{C}^{13}\text{OCH}_2\text{C}^{13}\text{OOH}$ , was submitted to this procedure; the results are given in Table I. The data leave no doubt concerning the correctness of this assumption. The acetate had the calculated  $\text{C}^{13}$  excess, and the formate, coming from the unlabeled  $\alpha$  position, had no excess  $\text{C}^{13}$ . The  $\text{C}^{13}$  content of the  $\text{CO}_2$  accurately reflected its origin from the carboxyl and partially from the  $\alpha$ -carbon, the observed and calculated values being 1.77 and 1.73 atom per cent respectively.

*Interference of  $\beta$ -Labeled Tyrosine*—It was also necessary to determine to what extent the presence of  $\beta$ -labeled tyrosine might interfere in this degradation procedure. Preliminary tests indicated that extensive oxidation of tyrosine occurred under the conditions of the oxidation but without the formation of appreciable amounts of volatile acids. To detect the possible formation of traces of these, 0.5 mm of non-isotopic acetoacetate was oxidized by permanganate in the presence of 12.5 mg. of radiotyrosine. The results, given in Table II, indicate that the  $\beta$ -carbon of tyrosine is not only oxidized to  $\text{CO}_2$  to an appreciable extent, but also



appears as formate or some substance chemically similar. Fortunately, however, no significant amounts of acetate are formed from the tyrosine  $\beta$ -carbon; hence there is no interference in the determination of the isotope content of the acetoacetate  $\beta$ - and  $\gamma$ -carbons.

With the two degradation procedures it was possible to obtain complete information concerning the  $C^{14}$  distribution in acetoacetate. The thermal decarboxylation (aniline citrate can also be used) provided a separation of the carboxyl from the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -carbons isolated as acetone, whereas the permanganate oxidation separated the  $\beta$ - and  $\gamma$ -carbons in the form

TABLE I

*Yields and  $C^{13}$  Distribution in Products Obtained by Permanganate Oxidation of Synthetic Sodium Acetoacetate Labeled with  $C^{13}$  in  $\beta$  and COOH Positions*

Over-all  $C^{13}$  excess = 1.32 atom per cent.

	Acetate		Formate		Carbon dioxide		
	Found	Calculated	Found	Calculated	Found	Calculated	Calculated on formate yield
Quantity, $\mu M$ .....	699	688	225	688	1040	688	1151
$C^{13}$ excess, atom %.....	1.28	1.32	0.00	0	1.77	2.64	1.73

TABLE II

*KMnO<sub>4</sub> Oxidation of 500  $\mu M$  of Non-Isotopic Acetoacetate in Presence of 12.5 Mg. of  $\beta$ -Labeled Tyrosine*

The specific activity of tyrosine = 100.

Product	Amount	Relative specific activity	Per cent of total activity
	$\mu M$		
Acetate.....	510	0.2	0.3
Formate.....	196	28	9
Carbon dioxide.....	1020	8	13

of acetic acid. A further separation of the  $\beta$ - and  $\gamma$ -carbons could be made by decarboxylation of the barium salt of acetate with recovery of the acetate carboxyl as barium carbonate; however, this step was not necessary in these experiments, since no activity was found in the acetate.

*$C^{14}$  Distribution in Acetoacetate Derived from  $\beta$ -Labeled Tyrosine*—With methods established for  $C^{14}$  distribution, two large scale experiments were carried out, each with 10 gm. of liver slices in 120 ml. of saline solution divided between four large Warburg flasks. After 2 hours of incubation, the solutions were combined, treated with copper-lime reagents, and the

filtrate divided into two portions. The first was used for recovery of acetone and  $\text{CO}_2$  by thermal decarboxylation as described previously; the second portion was submitted to oxidation with permanganate after addition of carrier acetoacetate. Data on  $\text{C}^{14}$  distribution are given in Table III; other over-all quantitative data for the same experiments are in Table IV.

TABLE III  
*Relative Specific Activities of Acetoacetate Breakdown Products*

Substance	Carbon of acetoacetate	Method of degradation	Specific activity	
			Experiment 2	Experiment 3
Acetone . . . . .	$\alpha, \beta, \gamma$	Thermal	31.6	41.5
Carbon dioxide . . . . .	COOH	"	0.7	0.4
Acetate . . . . .	$\beta, \gamma$	$\text{KMnO}_4$ oxidation	2.1	1.0
Formate . . . . .	$\alpha$	" "	187*	103*

\* Activity partly from residual tyrosine.

TABLE IV  
*Yields and Relative Activities of Acetoacetate and Respiratory  $\text{CO}_2$  from Experiments with  $\beta$ -Labeled Tyrosine*

Each experiment conducted with 10 gm. of liver slices in 120 ml. of saline solution. 2 hours at  $37^\circ$  in  $\text{O}_2$ .

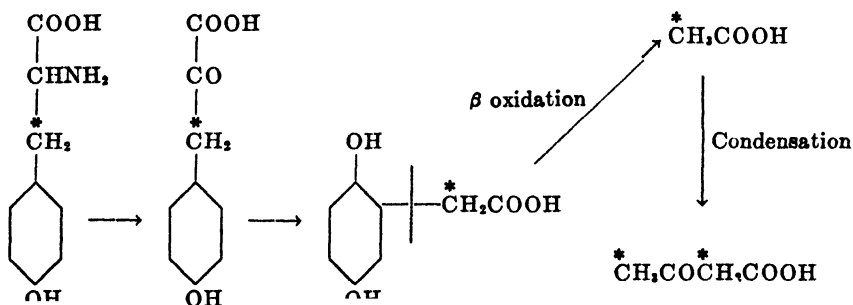
	Experiment 2		Experiment 3	
	Amount	Relative activity	Amount	Relative activity
	$\mu\text{M}$		$\mu\text{M}$	
Tyrosine added . . . . .	220	100	149	100
Acetoacetate . . . . .	166	24	150	31
Respiratory $\text{CO}_2$ . . . . .	1090	1.3	1270	0.8
Oxygen uptake . . . . .	1700		1990	

From Table III it is evident that essentially all of the acetoacetate radioactivity is in the  $\alpha$ -carbon. Neither the acetate, representing the  $\beta$  and  $\gamma$  positions, nor the  $\text{CO}_2$  obtained on thermal decarboxylation, thus representing the carboxyl position, had an appreciable activity.

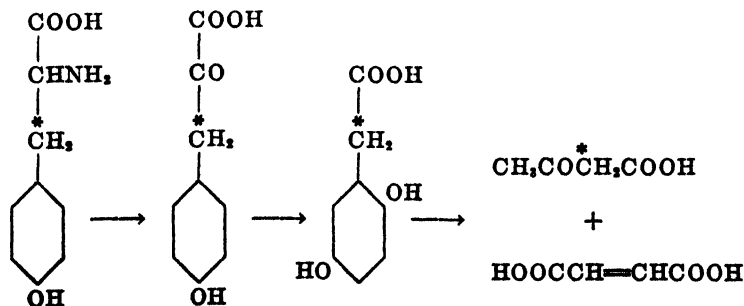
In all three experiments, radioactivity measurements were made on the residual washed tissue, and on the residues and extracts obtained therefrom after extraction with alcohol and ether. No radioactivity was found in these fractions, indicating that incorporation of  $\text{C}^{14}$  into the cell proteins or lipides was not appreciable under the conditions employed. Probably such incorporation could be shown with a more highly active tyrosine.

## DISCUSSION

Though the ketogenic activity of tyrosine and phenylalanine has been generally recognized for many years (7), actual proof for the conversion of tyrosine carbon to acetoacetate was obtained only quite recently (8). The present study establishes the liver as one of the sites of this transformation. Unlike ketogenesis from fatty acids, which involves a recondensation of 2-carbon units split off by  $\beta$  oxidation (4), ketogenesis from tyrosine apparently occurs via a different mechanism. As shown in the accompanying diagram, the condensation of 2-carbon units, split from the tyrosine side chain (presumably after conversion to homogentisic acid) would result in the formation of acetoacetate tagged in both the  $\alpha$ - and  $\gamma$ -carbons.

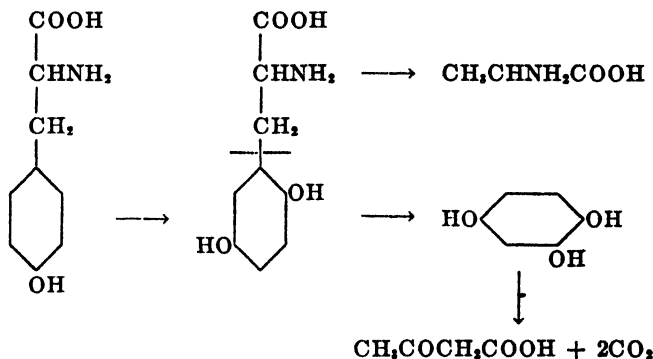


Restriction of  $\text{C}^{14}$  essentially to the  $\alpha$ -carbon indicates very strongly that the acetoacetate arises from an intact 4-carbon unit, and that two of the tyrosine ring carbons, as well as the  $\alpha$ - and  $\beta$ -carbons of the side chain, contribute to the acetoacetate molecule. Additional information concerning this process has recently been provided by Schepartz and Gurin (9) who found that phenylalanine, labeled in the 1 position of the benzene ring, yielded, in phlorhizinized rats, acetoacetate labeled mainly in the  $\gamma$ -carbon. This important finding not only completes the proof for participation of the ring carbons in acetoacetate formation, but also shows that the side chain migrates in homogentisic acid formation, as suggested many years ago by Neubauer and others (7).



Though some of the finer details of the reactions concerned in the conversion of tyrosine to acetoacetate are still in doubt, the data of this study are in full accord with a mechanism comprising the following steps: oxidative deamination (or transamination) to *p*-hydroxyphenylpyruvate; decarboxylation, oxidation, and side chain rearrangement to homogentisic acid; and oxidation of this diphenolic acid to acetoacetic and an as yet unknown 4-carbon residue, probably fumaric acid.

Felix and Zorn (10) have suggested that the breakdown of tyrosine involves the intermediate formation of  $\beta$ -(2,5-dihydroxyphenyl)alanine, which they consider yields alanine by hydrolytic cleavage of the side chain, together with a hydroxylated benzene ring, which breaks down to acetoacetate and 2 molecules of  $\text{CO}_2$ .



This hypothesis, which obviously is not supported by the isotopic data, is based on their observations that no ammonia is formed during the breakdown of L-tyrosine by pig liver minces, and that additional alanine appears in amounts roughly equivalent to the tyrosine metabolized. These findings are not necessarily inconsistent with the mechanism suggested by the isotopic data. If a 4-carbon dicarboxylic acid is formed from part of the tyrosine ring, it could yield pyruvate, and the transfer of the tyrosine nitrogen to pyruvate by transamination reactions conceivably could account both for the absence of ammonia and the extra formation of alanine.

**Rate of Tyrosine Oxidation in Liver**—In order to consider the quantitative significance of ketogenesis from tyrosine, data on the yields and specific activities of the acetoacetate and respiratory  $\text{CO}_2$  derived from these experiments are presented in Table IV. The following calculations from these data indicate that the acetoacetate derived from tyrosine makes up only a small portion of the total formed, and that the conversion of tyrosine to acetoacetate represents a very small fraction of the oxygen uptake of the liver slices.

On the basis of the mechanism established in these experiments it can be calculated that  $\beta$ -labeled tyrosine with an *over-all* activity of 100 would yield acetoacetate with an over-all activity of 225. This follows from the fact that the  $\beta$ -carbon has an activity of  $100 \times 9 = 900$  in tyrosine, and in acetoacetate it would be diluted by 3 non-isotopic carbons ( $900/4 = 225$ ). The acetoacetates formed in Experiments 2 and 3, respectively, had over-all specific activities (calculated from the acetone activities) of 24 and 31, which indicates that  $24 \times 100/225 = 10.7$  and  $31 \times 100/225 = 13.7$  per cent respectively of the total acetoacetate had its origin in tyrosine. Under similar conditions acetoacetate derived from labeled short chain fatty acids would constitute from 35 to 75 per cent of the metabolic acetoacetate.<sup>1</sup>

As to oxygen uptake, in Experiment 2 a total of  $166 \mu\text{M}$  of acetoacetate was formed, of which 10.7 per cent came from tyrosine. Thus  $166 \times 0.107 = 17.7 \mu\text{M}$  of acetoacetate came from tyrosine. If we assume the conversion takes place as follows:



it would have required  $5 \times 17.7/2 = 44 \mu\text{M}$  of oxygen, which is only  $44/1700 = 2.6$  per cent of the total oxygen uptake. In Experiment 3 the corresponding value is 5.1 per cent.

*Complete Oxidation of Tyrosine*—The very low recovery of  $\text{C}^{14}$  in the respiratory  $\text{CO}_2$  indicates that tyrosine is not metabolized to any significant extent by pathways leading to complete oxidation of the  $\beta$ -carbon. This observation is of additional importance in demonstrating the relative absence of ketolytic activity by the liver. Experiments thus far on fatty acid oxidation in liver have established two catabolic pathways, namely complete oxidation to  $\text{CO}_2$  and conversion to ketone bodies (4). It has been impossible, however, to determine with any certainty whether the metabolic  $\text{CO}_2$  arises by a pathway different from that which gives rise to ketone bodies or whether the  $\text{CO}_2$  is derived by the further oxidation of acetoacetate. If acetoacetate were readily oxidized by liver in these experiments, the  $\text{CO}_2$  of respiration would have had a relatively high specific activity, reflecting the activity of the acetoacetate. The low  $\text{C}^{14}$  content of the  $\text{CO}_2$  thus provides the first definite proof that the intact rat liver slice does not oxidize acetoacetate to an appreciable extent.

#### SUMMARY

When tyrosine, labeled with  $\text{C}^{14}$  in the  $\beta$  position, is incubated with liver slices of the rat, it is converted to acetoacetate labeled predominantly in the  $\alpha$  position. These results, in conjunction with those of other investi-

<sup>1</sup> Unpublished experiments.

gations, indicate that this conversion proceeds through the intermediate steps of *p*-hydroxyphenylpyruvic and homogentisic acids. This reaction is comparatively slow, and is not accompanied by other metabolic reactions of tyrosine involving the complete oxidation of the  $\beta$ -carbon.

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# A FRACTIONATION PROCEDURE FOR THE ACID-SOLUBLE PHOSPHORUS COMPOUNDS OF LIVER\*

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A procedure has been worked out for the fractionation of the acid-soluble P compounds of liver for use in tracer experiments. Like all similar fractionations, *e.g.*, those of LePage (6) and Kaplan and Greenberg (3), it is based principally on differences in the solubilities of the alkaline earth salts of these compounds and in their susceptibility to hydrolysis. LePage separates the "barium-insoluble" from the "barium-soluble" compounds, then applies appropriate analytical methods to each fraction. This is adequate for analytical purposes but does not effect the separation necessary for tracer experiments. Kaplan and Greenberg have carried the separation somewhat further in their tracer studies (4). The present procedure is considered to be an improvement over that of Kaplan and Greenberg in that a more complete separation of the alkaline earth-insoluble compounds from the soluble ones is achieved by the use of calcium in addition to barium than is possible with barium alone. Also, the alkaline earth-soluble fraction has been separated into several well defined entities.

The procedure is simpler than would be the case if phosphocreatine were present. Although a number of papers indicate the presence of this substance in liver, there are no reports of its isolation from this organ. When molybdic acid reagent was added at room temperature to liver filtrates from which inorganic P had been removed by magnesia mixture, no indication was obtained of the presence of any readily hydrolyzable P compound corresponding to phosphocreatine. The procedure as given below should be applicable to other mammalian tissues which do not contain phosphocreatine.

The fractionation has been applied to tracer studies on the metabolic significance of the phosphorus compounds, on rats of the Carworth and Sprague-Dawley strains. The results of these experiments will be described in a subsequent paper. It may be mentioned that differences were found between these two strains with respect to the amount of total acid-soluble P in the liver and in the amounts of adenylic acid, glucose-6-

\* This research was carried out at Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.



phosphate, and propanediol phosphate present. The analytical data of Table I were obtained on the livers of male rats of these two strains, weighing between 300 and 400 gm., fed *ad libitum* on Purina laboratory chow.

The liver was excised from the animal under pentobarbital anesthesia and immediately dropped into a freezing mixture, either liquid nitrogen or dry ice-ether. The frozen liver was powdered and extracted with approximately 5 volumes of ice-cold 10 per cent solution of trichloroacetic acid (TCA), in the manner previously described for muscle (14). The mixture was stirred for 15 minutes while being kept close to 0°, then filtered into a chilled receiver. The amount of filtrate obtained was usually such that 40 cc., representing 5 to 7 gm. of liver, were available for the fractionation. This generally contained 4 to 6 mg. of total acid-soluble P. The fractions that have been separated are inorganic orthophosphate, a nucleotide fraction consisting of the mixture of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) that is normally present in liver and from which the acid-labile P and acid-stable P have been separated, adenylic acid, glucose-1-phosphate, glucose-6-phosphate, phosphoglyceric acid, glycerophosphoric acid, propanediol phosphate, and a "coenzyme fraction" of which a major constituent is diphosphopyridine nucleotide (DPN). Some of the fractions are probably free of other compounds; others are known to be mixtures. This is particularly the case with the fractions derived from the material whose alkaline earth salts are water-soluble. On the average, some 80 per cent of the total acid-soluble P was accounted for in the several fractions.

The particulars of the procedure are given in the Flow Sheets 1 to 3 in sufficient detail so that the remaining discussion can be regarded in the nature of foot-notes to these flow sheets. The procedure is outlined in terms of working up 40 cc. of the TCA filtrate, the amount which would be available from the liver of a normal rat weighing from 300 to 400 gm.

The magnesia mixture used had the following composition: magnesium nitrate, 0.5 M; ammonium nitrate, 2 N; ammonium hydroxide, 2 N. Of this, 15 cc. were used for the precipitation of P as magnesium ammonium phosphate from 40 cc. or less of solution.

The molybdic acid reagent used was made by adding 1 volume of 20 per cent solution of ammonium molybdate in 1 N ammonium hydroxide to 1 volume of 7 N nitric acid. To each liter of the mixture was added 1 mg. of P as sodium phosphate. The material was let stand for at least 2 days and then filtered. For the precipitation of P as phosphomolybdate, the solution was made about 2 N with respect to nitric acid; 40 per cent solution of ammonium nitrate was added in an amount to give a final concentration of about 5 per cent, and 20 cc. of the molybdic acid reagent

then added. The solution was warmed to about 60° and let stand overnight before the precipitated phosphomolybdate was removed by filtration.

*Flow Sheet 1 (1)*<sup>1</sup>—The removal of glycogen from the TCA filtrate by precipitation with ethanol, first used by LePage (6), is essential, even when only traces of this substance are present. The small amounts extracted

#### FLOW SHEET 1

40 cc. TCA filtrate (1)

Add 45 cc. 95% ethanol; let stand overnight in refrigerator; centrifuge in refrigerated centrifuge

Ppt. Glycogen, discard	Supernatant (2) Add finely divided barium hydroxide to definite alkalinity to phenolphthalein, then 125 cc. ethanol; centrifuge immediately at room temperature
Ppt. Most Ba salts; starting material for Flow Sheet 2	Supernatant Contains coenzyme fraction and propanediol phosphate; let stand in refrigerator overnight, then centrifuge
Ppt. Coenzyme fraction Dissolve in dilute HNO <sub>3</sub> , evaporate on hot-plate to 5-10 cc., add 2 gm. NH <sub>4</sub> NO <sub>3</sub> , 10-15 cc. concentrated HNO <sub>3</sub> , digest on hot-plate 3-4 hrs., dissolve in about 50 cc. water, and add molybdic acid reagent to ppt. P of this fraction	Supernatant (3) Contains propanediol phosphate Acidify with acetic acid, concentrate to 10-15 cc., add equal volume concentrated HNO <sub>3</sub> , centrifuge; wash ppt. twice with 1:1 HNO <sub>3</sub>
Ppt. Ba(NO <sub>3</sub> ) <sub>2</sub> , discard	Supernatant and washings Add 2 gm. NH <sub>4</sub> NO <sub>3</sub> , digest on hot-plate 4-5 hrs., allowing most of acid to evaporate; add 50-60 cc. water and molybdic acid reagent to ppt. P of propanediol phosphate

by TCA from the livers of rats which had been fasted 24 hours were found to hold in solution as barium salts 1 to 2 mg. per cent of inorganic P and 3 to 5 mg. per cent of ATP-ADP as P. Simple dilution of the extracts, as recommended by Kaplan and Greenberg (3), is inadequate to overcome this interference.

<sup>1</sup> The figures in *italic* refer to the stages indicated in the flow sheets.

Only negligible amounts of P are lost by adsorption on the glycogen. This was shown by wet ashing the glycogen precipitates from four TCA filtrates. These were found to contain less than 20  $\gamma$  each of P out of the total of 4 to 6 mg. present in the TCA filtrates.

(2) The coenzyme fraction, so called by LePage (7), is a mixture of which DPN accounts for about one-half. This was shown by determination of the ribose phosphate ratios. The nature of the other substances present was not investigated. This fraction can be obtained only if the precipitated barium salts are separated by centrifugation almost immediately after the addition of the alcohol, as it begins to separate out at room temperature within a few hours.

(3) Propanediol phosphate, which Lindberg first discovered in the eggs of the sea-urchin and later isolated in brain and liver (10), is probably the only P compound present in the barium-soluble, alcohol-soluble fraction. The barium present was removed from the solution as nitrate rather than as sulfate, in order to minimize the possibility of losses by adsorption on  $\text{BaSO}_4$ . It is possible that some of the discrepancy between the amount of acid-soluble P present in the TCA filtrate and the sum of the fractions isolated may be accounted for by adsorption losses when it is necessary to remove barium as sulfate.

As may be seen in Table I, the range of values for this fraction in rats of the Carworth strain is of the order reported by Lindberg. In the Sprague-Dawley strain, two widely separate ranges were found, with no overlapping. The lower range is the same as in the Carworth rats, and 75 per cent of the values obtained was in this range. The upper range is almost 4 times as high.

*Flow Sheet 2* (4)—The addition of barium hydroxide to the TCA solution to pH 8.2, as applied by Cori and Cori (2) to muscle filtrates and recommended by LePage (6) as a general method, was found to leave in solution relatively large amounts of ATP-ADP and some inorganic P. On four filtrates which were treated in this way, the addition of the calcium salt to the supernatant precipitated inorganic P equivalent to 0.9, 1.3, 1.8, and 0.7 mg. per cent, and ATP-ADP equivalent to 5.9, 6.5, 1.9, and 4.2 mg. per cent of P. In four other filtrates to which barium hydroxide had been added in excess and the excess then neutralized with TCA, the calcium precipitates contained inorganic P equivalent to 0.1, 0.34, 0.26, and 0.3 mg. per cent, and ATP-ADP equivalent to 0.3, 1.0, 0.9, and 3.3 mg. per cent of P.

The omission of the calcium treatment might thus give an error of 50 per cent in the values for the glucose-1-phosphate fraction, since this is present to the extent of 1.7 mg. per cent as P, on the average, and the inorganic P remaining in the barium-soluble fraction would be precipitated

along with that of the glucose-1-phosphate. The ATP-ADP remaining in solution after barium would appear as part of the adenylic acid fraction.

TABLE I  
*Distribution of Acid-Soluble Phosphorus in Fractions Isolated from Livers of Normal Rats*

Values as mg. of P per 100 gm. wet weight of liver.

Fraction	Carworth strain, 18 rats		Sprague-Dawley strain, 20 rats	
	Mean and standard error of mean	Range	Mean and standard error of mean	Range
Total acid-soluble P*	77.3 ±2.3	53.9-88.0	84.1 ±2.2	66.1-99.6
Inorganic	18.5 ±0.6	13.1-23.9	19.2 ±0.65	14.4-26.4
Labile P of ATP-ADP	9.9 ±0.9	6.9-14.3	11.6 ±0.5	8.0-14.1
Stable P of ATP-ADP	6.1 ±0.4	4.9- 9.7	6.6 ±0.25	5.2- 8.8
Adenylic acid†	6.3 ±0.5	4.3- 7.3	4.1 ±0.3	2.3- 7.5
Phosphoglyceric acid	3.7 ±0.35	1.0- 6.9	3.2 ±0.1	2.2- 3.8
Glycerophosphoric acid	9.2 ±0.4	4.1-13.7	9.6 ±0.4	5.0-13.7
Glucose-1-phosphate	1.8 ±0.1	1.4- 3.1	1.7 ±0.15	1.1- 2.6
Glucose-6-phosphate‡	2.7 ±0.1	1.7- 3.3	3.8 ±0.3	2.8- 7.1
Coenzyme	3.1 ±0.4	1.4- 6.6	2.6 ±0.3	0.9- 5.3
Propanediol phosphate	1.6 ±0.2	0.7- 3.6	1.5 ±0.2‡ 6.1§	0.4- 2.5 5.1- 7.1
% of total accounted for in fractions, isolated	81	73 -93	80	71 -94

\* Difference between strains significant at level of  $p = 0.05$ .

† Difference between strains significant at level of  $p = 0.01$ .

‡ Three-fourths of group.

§ One-fourth of group.

(5) The ratio of acid-labile to acid-stable P in the ATP-ADP fraction was found to range from 1.2 to 2.0. The average value in both strains was 1.7, corresponding to 70 per cent ATP and 30 per cent ADP.

## FLOW SHEET 2

Ppt. of most Ba salts (4)

Dissolve in 20-25 cc. ice-cold 5% TCA; add powdered Ba(OH)<sub>2</sub> to marked alkalinity to phenolphthalein; adjust to pH 8.2 (faint pink) with TCA; let stand 15 minutes; centrifuge

Ppt. Ba-insoluble fraction with adsorbed Ba-soluble compounds; redissolve in 10-15 cc. ice-cold TCA, and reprecipitate with Ba(OH) <sub>2</sub> as above; centrifuge	Supernatant Part of Ba-soluble fraction with small amounts of Ba-insoluble compounds
Ppt. Most of Ba-insoluble compounds; reserve until Ca ppt. below is obtained	Supernatant Part of Ba-soluble fraction, with traces of inorganic P and ATP-ADP; add to supernatant above; add 2 cc. M solution of Ca trichloroacetate; let stand overnight in refrigerator; centrifuge
Dissolve in ice-cold TCA; combine with solution of Ca ppt.	Ppt. Traces of inorganic P and ATP-ADP; dissolve in ice-cold TCA; add to solution of Ba ppt.
To combined solutions above, add 1 cc. N H <sub>2</sub> SO <sub>4</sub> ; centrifuge immediately in refrigerated centrifuge; wash ppt. twice with ice-cold water	Supernatant Ba-Ca-soluble fraction; starting point for Flow Sheet 3
Ppt. BaSO <sub>4</sub> ; discard	Supernatant and washings (7) Add 15 cc. magnesia mixture; let stand 48 hrs. in refrigerator; filter
Ppt. Dissolve in 10 cc. dilute HNO <sub>3</sub> ; add molybdic acid reagent to ppt. inorganic P	Filtrate (7, 8) Aerate at 60° to remove ammonia (4-5 hrs.); make 1 N with HNO <sub>3</sub> ; heat 20 min. in bath of boiling water, cool, add excess ammonia; let stand 48 hrs. in refrigerator; filter
Ppt. Labile P of ATP-ADP (8); dissolve in dilute HNO <sub>3</sub> ; add molybdic acid reagent	Filtrate Contains stable P of ATP-ADP (as ribose-5-phosphate) and phosphoglyceric acid; add 4 cc. M Ba trichloroacetate; let stand overnight in refrigerator; centrifuge
Ppt. Digest with 2 gm. NH <sub>4</sub> NO <sub>3</sub> and concentrated HNO <sub>3</sub> 4-5 hrs. on hot-plate; add water, separate BaSO <sub>4</sub> at centrifuge; add molybdic acid reagent to supernatant to ppt. P of phosphoglyceric acid	Supernatant (8) Acidify with HNO <sub>3</sub> , evaporate to small volume, and digest with concentrated HNO <sub>3</sub> on hot-plate 3-4 hrs.; add sufficient water to dissolve Ba(NO <sub>3</sub> ) <sub>2</sub> ; then add molybdic acid reagent to ppt. stable P of ATP-ADP

Rapoport (11) reports that about 60 per cent, and LePage (7) that practically all, of this fraction in rat liver is ADP. Rapoport did not separate the ATP-ADP fraction from the adenylic acid, and his ratio is based on determinations by difference.

The customary hydrolysis period, 7 minutes, which is sufficient for small volumes of solution acidified with HCl, needed to be lengthened because of two factors. The relatively large volumes of solution dealt with require several minutes to reach the temperature of the water bath,

and the hydrolysis is slower in nitric acid than in HCl on account of the lower activity coefficient of the former. In previous studies on muscle extract with sulfuric acid, a 15 minute hydrolysis period was found necessary for small volumes of solution. For these reasons a 20 minute period was adopted in the present experiments.

(6) In the conversion of the stable P of this fraction to orthophosphate, removal of the barium present by precipitation as sulfate was found to result in large losses by adsorption. It is unnecessary to remove the barium, as the amount present as nitrate remains in solution and therefore does not interfere.

(7) It is necessary to allow all precipitations of magnesia mixture to stand in the refrigerator for 2 days to obtain as complete precipitation as possible. The necessity for this was established by the following experiment: Solutions containing tracer phosphate in the amounts arising from the fractionation were allowed to stand with magnesia mixture over 1 or 2 nights; the precipitates separated by filtration, and carrier phosphate equivalent to 1 mg. of P added to the filtrates. The carrier precipitates were removed by filtration the next day, and the original and carrier precipitates converted to phosphomolybdate. Determinations of radioactivity were then made. By this means it was found that, of 100 to 1000  $\gamma$  of P present as orthophosphate, 30 to 50  $\gamma$  remained in solution 1 day after the addition of the magnesia mixture. Allowing the precipitation to continue over a 2nd night reduced the amount remaining in solution to less than 10  $\gamma$ .

The 30 to 50  $\gamma$  remaining in solution might amount to 20 to 40 per cent of the P of glucose-1-phosphate. The appearance of this in the adenylic acid fraction would amount to a 5 to 15 per cent contamination of this fraction. Similarly, the P of adenylic acid remaining in solution after 1 night with magnesia mixture might amount to a 10 to 20 per cent contamination of the glucose-6-phosphate fraction. The 10  $\gamma$  amounts of P remaining in solution after 2 days standing with magnesia mixture might still amount to 10 per cent of the glucose-1-phosphate P, but would be less than 5 per cent contamination of the adenylic acid or glucose-6-phosphate fractions.

*Flow Sheet 3 (8)*—The adenylic acid fraction contains a few micrograms of P of the glucose-1-phosphate, and larger amounts derived from glucose-6- and fructose-6-phosphates. In addition, LePage (7) finds that there is some free pentose phosphate. Determinations of fructose by the method of Roe (12) indicate the presence of somewhat less than 0.5 mg. per cent of P as fructose-6-phosphate, and about this same amount of P is liberated by the hydrolysis of some 15 per cent of the glucose-6-phosphate. The analytical amount of P reported in this fraction is

## FLOW SHEET 3

Solution of Ba-Ca-soluble compounds from Flow Sheet 2; add 4 volumes 95% ethanol adjusted to pH 8.2, let stand overnight in refrigerator, centrifuge

<p>Ppt. Dissolve in 10 cc. 5 % TCA, add 1 cc. 1 N H<sub>2</sub>SO<sub>4</sub>, heat 10 min. in bath of boiling water, centrifuge, wash with water</p>	<p>Supernatant Discard</p>
<p>Ppt. BaSO<sub>4</sub></p>	<p>Supernatant and washings (7, 9) If aliquots are desired for determination of adenylic acid, fructose, glucose-1-phosphate P, make up to 25 cc., take aliquots for above determinations, and 20 cc. for pptn. of P of glucose-1-phosphate; otherwise, use entire solution for this pptn.; add magnesia mixture, let stand 48 hrs. in refrigerator, filter</p>
<p>Ppt. Dissolve in dilute HNO<sub>3</sub>; add molybdic acid reagent to ppt. P of glucose-1-phosphate</p>	<p>Filtrate (7, 8) Aerate at 60° to remove ammonia, make 1 N with HNO<sub>3</sub>, heat 5 hrs. in boiling water bath, cool, add excess ammonia, let stand 48 hrs. in refrigerator, filter</p>
<p>Ppt. Dissolve in dilute HNO<sub>3</sub>; add molybdic acid reagent to ppt. P of adenylic acid fraction</p>	<p>Filtrate Add 2 cc. M solution of calcium trichloroacetate, 4 volumes 95% ethanol; let stand overnight in refrigerator, centrifuge</p>
<p>Ppt. (10) Dissolve in 15 cc. 5% TCA, add NaOH to 1 N, heat 3 hrs. in bath of boiling water, cool, acidify to 2 N with nitric acid, add molybdic acid reagent, let stand overnight, filter</p>	<p>Supernatant Discard</p>
<p>Ppt. P of glucose-6-phosphate</p>	<p>Filtrate (11) Add 5 cc. 0.2 M HIO<sub>4</sub>, let stand 1 hr. at room temperature, bring acidity to 2.5-3 N with HNO<sub>3</sub>, heat 1 hr. in bath of boiling water. Ppt. is P of glycerophosphoric acid</p>

therefore erroneously high by some 15 to 25 per cent. The error in specific activity is relatively less important than the error in analytical amount, as the specific activities of the determinable contaminants were found to be so close to that of the adenylic acid P itself that the error was not significant.

Precipitation of adenylic acid by mercury, as carried out by Kaplan and Greenberg (3), might yield the adenylic acid with less contamination by other P compounds. However, LePage (8) found that in such a mercury precipitation not all of the P in the precipitate was associated with ribose. If mercury were used, the contaminants would then appear in the glucose-6-phosphate fraction. In this the error in analytical amount would be relatively greater than that arising from the hydrolysis procedure, and the possible error in determination of the specific activity might become appreciable. It was felt that mercury precipitation would not offer sufficient advantage to make up for the complications arising from its use.

(9) The solution is made up to volume and aliquots taken for determination of analytical amounts of adenylic acid, fructose-6-phosphate as fructose, and glucose-1-phosphate P. If these values are not desired, the entire quantity of solution obtained is used for the fractionation.

(10) The method for alkaline hydrolysis of glucose-6-phosphate is that of Kursanov (5), cited by Kaplan and Greenberg (3). It was found that, under the conditions described, more than 80 per cent hydrolysis was obtained. Assuming that the remainder was attacked by periodate in the subsequent treatment to obtain the P of glycerophosphoric acid, the maximum error in the analytical amount of this substance would be less than 5 per cent, and the error in specific activity would be negligible.

(11) The periodate oxidation of glycerophosphoric acid to the readily hydrolyzable glycolaldehyde phosphate was first used by Leva and Rapoport (9) as an analytical method. Since the alkali treatment for hydrolysis of the glucose-6-phosphate results in the formation of considerable of the  $\beta$  derivative, which does not react with periodate, the excess of this reagent is not removed. Heating the acid solution, as Leva and Rapoport have shown, results in rapid conversion of the  $\beta$  to the  $\alpha$  form, which then undergoes the oxidation and hydrolysis. On pure  $\alpha$ -glycerophosphate, and on material first heated with 1 N NaOH, 93 per cent yields were obtained, compared to the 96.5 per cent reported by Leva and Rapoport. This slight difference may be due to the higher acidity present than is prescribed for the analytical method.

For determination of radioactivity, the phosphomolybdate precipitates obtained were collected in Pyrex Gooch crucibles with sintered glass disks, modified either for use with a thin walled Geiger-Müller counter or, in the later experiments, for counting with the methane flow proportional



counter of Bernstein and Ballentine (1). The precipitates in the crucible were washed with 0.2 per cent solution of Aerosol MA in 5 per cent ammonium nitrate to overcome the tendency to creep. After uniform distribution over the surface of the disk had been obtained by this means, the Aerosol and ammonium nitrate were washed out with ethanol, and the samples allowed to dry at room temperature. This was sufficient preparation for use with the Geiger-Müller tube. With the methane flow counter, however, it was found necessary to place 0.5 cc. of a 1 per cent solution of collodion in acetone in the crucible and allow the acetone to evaporate at room temperature. The collodion film thus formed keeps the precipitate from being disturbed by the current of methane.

After the counting, the phosphomolybdate is dissolved by the addition of 1 cc. of phosphate-free 1 N NaOH. The solution and washings are made up to volume and suitable aliquots taken for determination of P by the method of Fiske and Subbarow. The modifications of the Gooch crucible and the other apparatus used for the filtration and solution of the precipitate have been described elsewhere (13).

The author is indebted to the generosity of Dr. Carl Neuberg and of Dr. G. A. LePage for supplying the samples of 3-phosphoglyceric acid and glucose-6-phosphate, respectively, which were used in working out the procedures described for the isolation of the P of these fractions.

#### SUMMARY

1. A procedure has been described for the fractionation of the acid-soluble P compounds of liver for tracer experiments.
2. The P of the following fractions is probably free, or practically free, of other P compounds: inorganic P, ATP-ADP, phosphoglyceric acid, glycerophosphoric acid, propanediol phosphate, glucose-1-phosphate, and glucose-6-phosphate.
3. A fraction containing principally adenylic acid and one of which the major constituent is DPN have also been obtained. These are known not to represent single P compounds.
4. The procedure has been applied to the livers of rats of the Carworth and Sprague-Dawley strains, and some differences have been found between these strains with respect to the amounts of P in the adenylic acid and glucose-6-phosphate fractions and in the total acid-soluble P.
5. In the livers of the rats of the Sprague-Dawley strain, the amounts of propanediol phosphate present have been found to fall into two distinct ranges, of which the higher is about 4 times the lower. The amounts of this substance found in the livers of the Carworth strain animals are in the lower range.

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## QUINOLINIC ACID METABOLISM

### I. URINARY EXCRETION BY THE RAT FOLLOWING TRYPTOPHAN AND 3-HYDROXYANTHRANILIC ACID ADMINISTRATION

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The presence of an acid-labile precursor or derivative of nicotinic acid in the urine of rats following the ingestion of large amounts of tryptophan was first reported by Singal *et al.* (1). Using a number of adsorbing agents, they concentrated the substance by adsorption from acid solution and elution with dilute alkali. It was pointed out that this compound resembled quinolinic acid with regard to stability to heat at various pH values and that their concentrates gave a positive color test with ferrous sulfate, as does quinolinic acid. The suggestion was made that quinolinic acid might be formed from an indole nucleus through a quinoline derivative, since kynurenic acid can arise from tryptophan (2).

Mitchell and Nyc (3) reported experiments which indicate that 3-hydroxyanthranilic acid is an intermediate in the transformation of tryptophan to nicotinic acid in *Neurospora*. This compound also has nicotinic acid activity for the rat (4). Quinolinic acid is a likely intermediate between 3-hydroxyanthranilic acid and nicotinic acid, since it could be formed by the oxidative cleavage of the benzenoid nucleus in the 3-4 position followed by reclosure with the amino group. Evidence for this reaction has been obtained and will be presented in this and subsequent communications.

A preliminary report (5) from this laboratory described the isolation of the "acid-labile" form of nicotinic acid from rat urine and its identification as quinolinic acid. The details of these studies are reported here.

#### EXPERIMENTAL AND RESULTS

*Care of Animals*—Adult male rats from the stock colony were placed in individual metabolism cages over glass funnels and fed the 9 per cent casein ration (6) which has been widely used for tryptophan-nicotinic acid studies. After 3 weeks, a 3 day collection of urine was taken to establish preexperimental control values. The ration was then supplemented with 2.5 per cent DL-tryptophan at the expense of the sucrose and fed *ad libitum* while urine was collected under toluene for five 3 day periods. The average food consumption was 8.8 gm. per rat per day, as compared to 10

to 13 gm. per day in the preexperimental period; all animals lost weight during the period of tryptophan administration. In preliminary studies adult animals of similar history and age were employed with the same results as in the experiment reported in detail here.

*Methods*—A modification<sup>1</sup> of the *Lactobacillus arabinosus* assay for nicotinic acid (7, 8) was used. A number of known metabolites of tryptophan were tested for growth-promoting activity under the conditions of the assay. The results<sup>2</sup> showed that tryptophan, kynurenine, kynurenic acid, xanthurenic acid, 1-methyl-6-pyridone-3-carboxamide, *N*<sup>1</sup>-methylnicotinamide, 3-hydroxyanthranilic acid, and pyridine-3,6-dicarboxylic acid possessed no biological activity and none resulted when they were autoclaved for 2 hours with glacial acetic acid. A number of these compounds have been tested by various investigators and found inactive for *L. arabinosus*.

A chemical method for the determination of nicotinic acid, in which cyanogen bromide and aniline were employed (9), was used for following the progress of fractionation procedures in some cases. Quinolinic acid did not give a color with these reagents but led to the expected color intensity following decarboxylation by autoclaving for 2 hours with glacial acetic acid.

*Production of Nicotinic Acid Activity in Urine*—Singal *et al.* (1) reported excretion values for the "acid-hydrolyzable" form of nicotinic acid, using the microbiological assay following autoclaving with 1 N sulfuric acid for 15 minutes at 15 pounds pressure. Apparently the rate of the reaction involved was not determined. In our studies the effects of acid concentration and time on the formation of the active compound were first determined. Urine from rats receiving DL-tryptophan was used. It was found (Table I) that the maximum values for the urine specimens studied were not reached until the autoclaving with 1 N hydrochloric acid had been continued for approximately 24 hours. While the time required to obtain maximum values varied slightly from one experiment to the other, in all cases more than 15 hours were required; 10 to 12 per cent was released in 1 hour and approximately 40 per cent in 4 hours. From 7 to 8 per cent decarboxylation of quinolinic acid resulted when it was autoclaved for  $\frac{1}{2}$  hour with 1 N sulfuric acid. The production of activity in urine with 0.1 N and 6 N hydrochloric acid was slower than with 1 N acid. 4 hours of autoclaving with 6 N hydrochloric acid or 1 hour with 5 N sodium hydrox-

<sup>1</sup> Unpublished.

<sup>2</sup> These compounds were kindly supplied by the following: Dr. S. Lepkovsky, Division of Poultry Husbandry, University of California (kynurenine, kynurenic acid), Dr. B. S. Schweigert, American Meat Institute Foundation, Chicago (xanthurenic acid), Dr. B. C. Johnson, Department of Animal Nutrition, University of Illinois (1-methyl-6-pyridone-3-carboxamide), Dr. H. A. Lardy, Department of Biochemistry, University of Wisconsin (pyridine-3,6-dicarboxylic acid).

ide did not result in destruction of the active compound, since complete activity was found after subsequent treatment with 1 N hydrochloric acid. These results suggested that the reaction involved in the production of a compound possessing nicotinic acid activity for *L. arabinosus* is non-hydrolytic.

Other reagents were tested, and glacial acetic acid was found to be very effective. Maximum values, which correspond to those obtained with prolonged autoclaving with 1 N hydrochloric acid, were obtained when the urine was autoclaved with glacial acetic acid for 1 to 2 hours. The rates of decarboxylation of pure quinolinic acid under the influence of 1 N hydro-

TABLE I

*Effect of Reagents and Time of Autoclaving on Production of Nicotinic Acid from Quinolinic Acid and Rat Urine*

All values are expressed as micrograms of nicotinic acid activity per ml. for *L. arabinosus* and are corrected for active compounds present in urine before autoclaving.

Time	Rat urine, 125 ml. per rat per day		Quinolinic acid, 100 gamma per ml.	
	1 N hydrochloric acid	Glacial acetic acid	1 N hydrochloric acid	Glacial acetic acid
<i>hrs.</i>				
0	2.7		0.009	
$\frac{1}{2}$		63.3		60.0
$\frac{1}{4}$		75.8		66.5
1	8.3	75.1		72.0
2	15.1	83.3	13.0	71.6
4	33.8	83.3	31.2	68.3
8	61.0			
16	76.0			
24	82.0			
48	82.7			

chloric acid and glacial acetic acid at 15 pounds pressure agreed well with the corresponding rates of formation of nicotinic acid in the urine of tryptophan-fed rats.

Prior to the feeding of tryptophan the rats excreted approximately 30  $\gamma$  per day of "free"<sup>a</sup> nicotinic acid and a considerable amount of the "acid-labile" form. During the feeding of the tryptophan-containing ration the "free" nicotinic acid rose to 300 to 500  $\gamma$  per day and the total activity after acetic acid treatment to 6 to 11 mg. per day in the different collection periods. A more detailed study of the effect of diet on urinary excretion of quinolinic acid, nicotinic acid, and *N*<sup>1</sup>-methylnicotinamide has been made (10).

<sup>a</sup> Microbiologically available.

*Isolation*—The urine from four rats (15 days collection) was diluted to 2500 ml., adjusted to pH 1.4 with hydrochloric acid, and stirred for 1 hour with 100 gm. of norit. After standing at room temperature for 2 hours, the norit was filtered off and washed with 100 ml. of distilled water. The active material was eluted by stirring for 1 hour with 2 liters of 0.1 N ammonium hydroxide. After standing for 1 hour, the norit was removed by filtration and discarded. These procedures resulted in the recovery of more than 80 per cent of the activity with approximately 20 per cent of

TABLE II  
*Progress of Isolation of Quinolinic Acid from Rat Urine*

Fraction No.	Description	Dry weight	Nicotinic acid activity	
			Untreated (free nicotinic acid)	Autoclaved with acetic acid (nicotinic acid + quinolinic acid)
		gm.	mg.	mg.
1	Urine	30 Ca.	27	490
2	Norit filtrate	24.35	3.4	8.6
3	" eluate	6.38	5.5	408
4	Acid methanol residue	0.323	0.075	4.8
5	" " extract placed on column	6.06	5.85	380
6	Methanol washings		0.16	1.76
7	Water washings		0	0.2
8	1st NH <sub>4</sub> OH fraction, to pH 8.9		5.1	9.0
9	2nd " " " 8.9-9.2	0.078	0	29.8
10	3rd " " " 9.2-9.3	0.912	0.136	323
11	4th " "	0.225	0	10.1
12	After Amberlite treatment of Fraction 10			260
13	Ether extract of Fraction 12	0.006		0.56
14	Crystalline product from 40% acetic acid	0.207		153

the solids (Table II). Some experiments indicated that approximately 10 per cent more activity could be recovered by eluting with ammonium hydroxide a second time.

After removal of small aliquots for analysis, the eluate was concentrated to 50 ml. *in vacuo* at a temperature below 40° and extracted twice with equal volumes of warm chloroform. The chloroform extract contained no activity and was discarded. The aqueous solution was then concentrated to dryness *in vacuo* and extracted with 40 ml. of warm methanol after being adjusted to pH 3.0 by the dropwise addition of concentrated hydrochloric acid. A small amount of a white, crystalline, water-soluble

solid which did not dissolve in the methanol was filtered off and found to contain little active material (Fraction 4, Table II).

The acid-methanol extract was chromatographed on a 30 mm. column containing 250 gm. of activated alumina.<sup>4</sup> 800 ml. of methanol were used to wash, followed by 2050 ml. of distilled water (Fractions 6 and 7, Table II). The column was then developed with ammonium hydroxide prepared by diluting 1 ml. of concentrated ammonium hydroxide to 500 ml. with distilled water. The activity in the various fractions as estimated by the ferrous sulfate test (11) agreed with that obtained by microbiological assay following decarboxylation. Noting the pH changes was also useful, since there was a rather rapid increase as the column was developed, until the active compound appeared in the eluate at pH 9 to 9.2. This pH range corresponds to that at which the bivalent anion of quinolinic acid is formed, as shown by an experimentally determined dissociation curve of quinolinic acid. During the elution of the active compound the pH remained constant, but it rose rapidly in the inactive fractions which followed. Authentic quinolinic acid behaved in the same manner on a similar alumina column.

Fraction 10, which contained only a trace of "free" nicotinic acid, accounted for 323 mg. of the 380 mg. of activity in the methanol extract chromatographed. This fraction was concentrated to dryness *in vacuo* and fern-like crystals formed. The dry weight corresponded to a preparation of the diammonium salt of quinolinic acid which was 58 per cent pure, with retention of 70 per cent of the original activity.

This fraction in 40 ml. of distilled water was treated with 12 gm. of Amberlite IR-100-H and, after stirring for 3 to 5 minutes, at which time the pH was 2.3, the resin was filtered off and washed with small portions of water to give a total volume of filtrate and washings of 200 ml. 260 mg. or 80 per cent of the activity survived this step.

The aqueous solution of free quinolinic acid was extracted with an equal volume of ether. The water layer was concentrated to dryness *in vacuo*, and the residue dissolved in 9 ml. of hot 40 per cent acetic acid. On cooling, 207 mg. of white crystals appeared. They were removed and recrystallized from 40 per cent acetic acid.

*Identification*—On the micro block<sup>5</sup> the isolated compound began to sublime at 166°, evolved a gas at 188–190°, and formed new crystals which melted above 230°. When heated rapidly, it melted completely at 232–237° with sublimation. Authentic quinolinic acid alone or mixed with

<sup>4</sup> Alcoa activated alumina grade No. 20, 80 to 200 mesh, was treated with concentrated hydrochloric acid for 1 hour, washed until chloride-free, and heated to 250–280° for 1 hour.

<sup>5</sup> All melting points were taken on the Kofler micro block and are corrected.

the isolated compound sublimed at 166–168°, suddenly changed crystalline form, with the evolution of gas, at 188–190°, and melted at 227–230° with rapid sublimation.

$C_7H_5O_4N$ . Calculated. C 50.28, H 3.02, N 8.39  
Found. " 50.52, " 3.17, " 8.44

Further proof of the identity of the compound with quinolinic acid was provided by comparison of the ultraviolet absorption spectrum with that of pure quinolinic acid and by the preparation of derivatives.

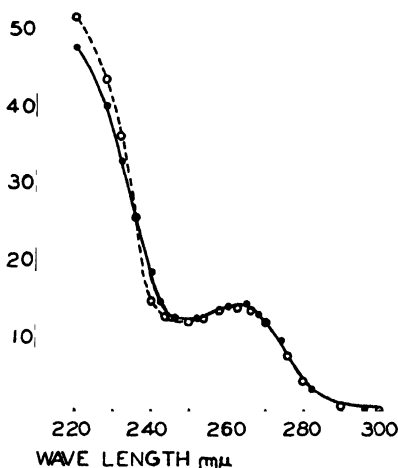


FIG. 1. Ultraviolet absorption spectra of authentic quinolinic acid (●) and that isolated from urine (○) in 95 per cent ethanol.

$$k = \frac{\log_{10} I_0/I}{\text{gm. per liter} \times \text{length in cm.}}$$

In Fig. 1 are shown the ultraviolet absorption spectra for the compound isolated and authentic quinolinic acid. The two curves coincide over a considerable portion of the range measured.

5 mg. of the isolated quinolinic acid were autoclaved for 1½ hours with 0.5 ml. of glacial acetic acid. The acetic acid was removed and the nicotinic acid recrystallized from ethanol. The product melted at 230–234° with sublimation when heated rapidly, and there was no depression of the melting point when the product was mixed with authentic nicotinic acid.

The dimethyl esters of the compound from rat urine and authentic quinolinic acid were prepared as follows: 10 mg. of each were dissolved in 5 ml. of absolute methanol and 0.2 ml. of acetyl chloride was added. After 12 hours in a desiccator over phosphorus pentoxide, at room temperature, the material was evaporated to dryness *in vacuo*. 3 ml. of distilled water



were added and the pH adjusted to 8.5 with solid sodium bicarbonate. The esters were removed by extraction with two 5 ml. portions of chloroform. After drying with anhydrous magnesium sulfate, the chloroform was removed. The esters were recrystallized twice from a mixture of carbon disulfide and Skellysolve B. The birefringent plates melted at 53–55°; the ester prepared from quinolinic acid melted at 53–54° and the mixed melting point was 53–54.5°.

#### DISCUSSION

The marked increase in the amount of quinolinic acid in the urine following tryptophan feeding is interpreted as evidence that this compound is formed from tryptophan. Unequivocal proof of this conversion can only be provided by studies with isotopically labeled tryptophan. If tryptophan is a true precursor of quinolinic acid, it would be of interest to know whether the latter is an intermediate in the now established conversion of tryptophan to nicotinic acid (12) or a side reaction product. The low activity of quinolinic acid for the growth of the rat and *Neurospora* (10), the excretion of large amounts of quinolinic acid following the injection of 3-hydroxyanthranilic acid (10), the accumulation of quinolinic acid in the medium of a nicotinic acid-requiring mutant of *Neurospora* (10), and the conversion *in vitro* of 3-hydroxyanthranilic acid to quinolinic acid by rat liver slices or homogenates (13, 14) are all consistent with the postulate that quinolinic acid is an intermediate.

There have been a number of observations reported which suggest that quinolinic acid is capable of replacing nicotinic acid for various living organisms, presumably by decarboxylation to nicotinic acid. Dann *et al.* (15) found that quinolinic acid exhibited variable though definite preventive action for blacktongue in dogs. In curative tests it was found inactive (16, 17). 1 gm. doses of quinolinic acid improved the condition of pellagrins (18), suggesting that in man decarboxylation can occur. Koser *et al.* (19) have suggested that the activity reported for man and the dog may be due to the nicotinic acid present as impurity.

Quinolinic acid has been reported to possess nicotinic acid activity for *Proteus* (20, 21), but little or none for *Lactobacillus arabinosus* (21), *Staphylococcus aureus* (22, 23), *Torula cremoris* (24), dysentery bacilli (19), or *Neurospora* (3, 25). While the variation in results may be due in part to different degrees of purity of the quinolinic acid used and to variations in the extent of decarboxylation occurring during autoclaving, these do not appear to provide a complete explanation for the activity observed. Dann *et al.* (15) found, by a chemical method, less than 0.03 per cent nicotinic acid in the sample they used, which would not account for the antiblacktongue activity noted. Ellinger and coworkers (21) found from 12 to 25

per cent of the expected activity with various species of *Proteus*, while 1.2 per cent activity was found with *L. arabinosus*, suggesting that in the former case, at least, decarboxylation by the organism did occur. Except for the report that *N*<sup>1</sup>-methylnicotinamide is excreted by the rat following the administration of quinolinic acid (21), no previous studies of the activity for this species have been reported.

#### SUMMARY

1. Rats receiving a 9 per cent casein-low niacin diet, supplemented with 2.5 per cent DL-tryptophan, excrete quinolinic acid at the rate of 6 to 11 mg. per rat per day.

2. The acid was isolated by adsorption on and elution from norit and chromatographing on alumina from methanol with the aid of dilute ammonium hydroxide as a developing solvent. The free acid was crystallized from 40 per cent acetic acid.

3. It was identified by melting point, analysis, ultraviolet absorption spectrum, preparation of the dimethyl ester, and decarboxylation to nicotinic acid. The latter was identified by melting point, mixed melting point, the cyanogen bromide-aniline color reaction, and biological activity for *Lactobacillus arabinosus*.

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## QUINOLINIC ACID METABOLISM

### II. REPLACEMENT OF NICOTINIC ACID FOR THE GROWTH OF THE RAT AND NEUROSPORA

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Since the observation (1) that tryptophan and nicotinic acid are mutually interchangeable, within limits, in supporting the growth of rats, considerable evidence has accumulated which indicates that biosynthesis of nicotinic acid can proceed from tryptophan (2-5). Unequivocal evidence for this transformation was provided by the experiments of Heidelberger *et al.* (6) who fed labeled tryptophan ( $\beta$ -indolyl-3- $C^{14}$ - $\alpha$ -aminopropionic acid) and found that the  $N^1$ -methylnicotinamide isolated from the urine contained  $C^{14}$  in the carboxamide group.

Studies with *Neurospora* have demonstrated a similar synthesis (7, 8) involving kynurenine and 3-hydroxyanthranilic acid as intermediates. That the synthesis proceeds by this pathway in the rat is suggested by the demonstration (9) that 3-hydroxyanthranilic acid replaces tryptophan or nicotinic acid for growth in the rat and increases the excretion of nicotinic acid in the urine (10).

Recently work from this laboratory (11, 12) has shown that quinolinic acid is excreted by the rat following tryptophan or 3-hydroxyanthranilic acid administration. While this suggests that quinolinic acid is another intermediate, formed by oxidative cleavage of the benzenoid nucleus of 3-hydroxyanthranilic acid, quinolinic acid has been reported inactive for the growth of *Neurospora crassa* mutants (7, 9).

The studies reported here concern the ability of quinolinic acid to replace nicotinic acid in the diet of the rat and the effects of the incorporation of the probable intermediates into the diet on the urinary excretion of nicotinic acid,  $N^1$ -methylnicotinamide, and quinolinic acid. The failure of quinolinic acid at low levels to support the growth of *N. crassa* has been confirmed. At higher levels, however, mutant 4540 responded, while mutant 3416 did not. Quinolinic acid has been isolated from the medium of mutant 3416 following growth with limiting amounts of nicotinamide.

#### EXPERIMENTAL AND RESULTS

**Methods**—All rats used for growth studies were obtained from the stock colony at approximately 21 days of age. They were selected to give even

distribution of litter mates in each experimental group and were fed the experimental rations *ad libitum* for 4 or 5 weeks. The basal ration was identical to that described by Hankes *et al.* (13) with 0.2 per cent L-cystine and 0.2 per cent DL-threonine. Supplements were given by (1) daily injection intraperitoneally, (2) as a small volume of solution in a supplement dish once daily, or (3) mixed in the ration.

Nicotinic acid values as used here refer to those obtained by analysis of urine without preliminary treatment. They may include nicotinic acid, nicotinamide, nicotinuric acid, and any other compound active for *Lactobacillus arabinosus*. It has been found that mild alkaline hydrolysis of rat urine does not alter the nicotinic acid available to *Lactobacillus arabinosus*, which confirms the observations of Singal *et al.* (4). Quinolinic acid values were calculated from the difference between the nicotinic acid content before and after autoclaving with glacial acetic acid. While this value may include other compounds which acquire nicotinic acid activity during autoclaving with acetic acid, it has been considered as representing only quinolinic acid, since no evidence has been obtained for the presence of other such compounds in rat urine (12).

An appropriate volume of rat urine was evaporated to dryness in a test-tube or 50 ml. Erlenmeyer flask on a steam bath or at low temperature *in vacuo*. To the dry residue, 0.5 ml. of glacial acetic acid was added and the receptacle autoclaved for 2 hours at 15 pounds pressure. Periods as short as 1 hour with as little as 0.2 ml. of acetic acid appear adequate, though complete decarboxylation is assured by the former treatment. The samples were then neutralized to pH 7.0 with sodium hydroxide and diluted to a volume such that the nicotinic acid content was approximately 0.1  $\gamma$  per ml. The microbiological determinations were made with *L. arabinosus* by use of either turbidimetric or titrimetric procedures with a total volume of 2 ml. per tube. Most values reported are based on the results obtained with twelve to eighteen tubes per sample. *N*<sup>1</sup>-Methylnicotinamide was determined by the acetone-fluorometric method of Huff and Perlzweig (14).

The 3-hydroxyanthranilic acid was prepared from 3-methoxybenzoic acid by the method of Nyc and Mitchell (15). Quinolinic acid was prepared by oxidizing 8-hydroxyquinoline with nitric acid (16). The quinolinic acid used in the first two experiments contained 0.009 per cent of nicotinic acid as measured microbiologically, and that used in the other experiments contained less than 0.002 per cent. It was not possible to determine precisely the amount of nicotinic acid present in the latter sample with the microbiological method because non-competitive inhibition prevented the addition of sufficiently high levels of the sample to promote maximum growth.

*Excretion Studies with Adult Rats*—Four male rats weighing 286 to 323

gm. were fed the basal ration without added threonine for 9 days. They were then placed in individual metabolism cages and, while the same regimen was continued, urine was collected for two consecutive 48 hour periods. Each of two animals then received 2 mm of L-tryptophan, intraperitoneally, during a 48 hour period, in ten 5 ml. portions of 0.9 per cent sodium chloride solution at pH 6. The other two rats received, in the

TABLE I  
*Urinary Excretion of Metabolites by Rat Following Intraperitoneal Administration of Tryptophan and 3-Hydroxyanthranilic Acid*

Rat No.	Metabolite* excreted	Collection period†				
		A	B	C	D	E
		mg.	mg.	mg.	mg.	mg.
1	Nicotinic acid	0.020	0.017	0.26	0.45	0.040
	Quinolinic acid	0.111	0.067	14.3	23.8	0.45
	N <sup>1</sup> -Me‡	0.065	0.050	6.3	13.0	0.93
2	Nicotinic acid	0.025	0.021	0.20	0.27	0.045
	Quinolinic acid	0.049	0.049	17.3	9.5	0.20
	N <sup>1</sup> -Me	0.045	0.050	3.6	5.2	0.78
3	Nicotinic acid	0.026	0.023	0.095	0.27	0.064
	Quinolinic acid	0.064	0.051	2.1	17.9	1.44
	N <sup>1</sup> -Me	0.115	0.080	1.6	5.8	1.95
4	Nicotinic acid	0.021	0.016	0.11	0.26	0.038
	Quinolinic acid	0.056	0.037	3.4	28.9	0.82
	N <sup>1</sup> -Me	0.10	0.10	1.74	5.1	0.78

\* Nicotinic acid was measured by the microbiological method without preliminary treatment of the urine. Quinolinic acid values represent the increase in nicotinic acid value on autoclaving with acetic acid; to convert to quinolinic acid multiply by 1.36.

† Collection Period A was the 3rd and 4th days prior to the administration of the compounds; Period B, the 2 days immediately preceding; Period C, the first 24 hours of administration; Period D, the second 24 hours; and Period E, the 2 days immediately following. All values are for 24 hour excretion. Rats 1 and 2 received 1 mm (204 mg.) of L-tryptophan per day for 2 days (during Periods C and D). Rats 3 and 4 received a corresponding amount (153 mg.) daily of 3-hydroxyanthranilic acid.

‡ N<sup>1</sup>-Methylnicotinamide analyses were kindly done by Dr. G. B. Ramasarma.

same manner, 2 mm of 3-hydroxyanthranilic acid in saline suspension, adjusted to pH 5.5 to 6.5. 24 hour urine collections were made during this 2 day period and a fifth collection was made during the 2 following days. All urine collections were carried out under toluene. The urine was analyzed for nicotinic acid, quinolinic acid, and N<sup>1</sup>-methylnicotinamide (N<sup>1</sup>-Me) as described above.

During the preinjection periods (A and B, Table I) all rats excreted

small and relatively constant amounts of all three metabolites. When tryptophan was administered (Rats 1 and 2, Periods C and D), the urinary excretion of nicotinic acid rose about 10-fold,  $N^1$ -Me about 100-fold, and quinolinic acid about 200- to 300-fold. A rather prompt return to normal values resulted following the cessation of tryptophan injections. The injection of 3-hydroxyanthranilic acid caused a similar elevation in the urinary excretion of these metabolites, but the response appeared to be delayed. There was also some evidence of greater delay in the return to normal values. This may reflect delayed absorption of the sparingly soluble 3-hydroxyanthranilic acid, which could be administered at such dosages only in suspension. The quinolinic acid excretion was from 20.0 to 38.0 mg., expressed as nicotinic acid, during the 2 day period, accounting for 8 to 15.5 per cent of the total material administered, while  $N^1$ -methylnicotinamide accounted for 2.5 to 8 per cent. Rat 1 excreted approximately 23 per cent of the administered tryptophan in the form of these two metabolites.

From these results it is evident that both in the control period and during the injection of precursors of nicotinic acid quinolinic acid is quantitatively at least as important as  $N^1$ -Me as an excretion product. These results confirm the intermediary rôle of 3-hydroxyanthranilic acid which had been indicated by growth and excretion studies (9, 10). Additional evidence that tryptophan and 3-hydroxyanthranilic acid increase the excretion of quinolinic acid is presented in the following section.

*Quinolinic Acid and 3-Hydroxyanthranilic Acid for Rat Growth*—Four growth studies were conducted in an effort to determine the relative ability of tryptophan, 3-hydroxyanthranilic acid, quinolinic acid, and nicotinic acid to supplement a nicotinic acid-tryptophan-deficient diet. In Experiment I (Table II) the first three of these metabolites were administered once daily by intraperitoneal injection of 0.02 mm of each in 1 ml. of 0.9 per cent sodium chloride. Three female rats were used in each group; all received the threonine-containing basal ration *ad libitum*. All compounds produced marked improvement in the growth rates. 3-Hydroxyanthranilic acid appeared to be slightly more effective than the other supplements, since all three animals grew at a more rapid rate than any of the animals in the other groups. This may have been the result of the gradual availability of this compound, which was administered as a suspension. Quinolinic acid appeared to be slightly inferior to the other substances, which is not surprising in view of the extent to which it was excreted unchanged. In this experiment the average quinolinic acid excretion with a relatively low level of intake was 2.62 mg. or 80 per cent of that injected.

The studies of urinary excretion reported in Table II were made on individual collections in two 3 day periods during the 4th week of the experi-



ment. Individual analyses were made on each of these twenty-four samples, but only the average values with standard errors are shown in Table II.

In Experiment II, 0.02 mm of the quinolinic acid was administered daily as a solution in a supplement dish to female weanling rats. The entire dose was consumed promptly. By this route of administration quinolinic acid again showed a marked effect on the growth.

In other experiments (Table III) attempts were made to determine the relative effectiveness of dietary nicotinic acid, quinolinic acid, and trypto-

TABLE II

*Growth and Urinary Excretion of Nicotinic Acid, Quinolinic Acid, and N<sup>1</sup>-Methylnicotinamide by Rat As Influenced by Precursors of Nicotinic Acid*

Experi- ment No.	Group No.	Supplement	Average growth, 4 wks.	Urinary excretion, average of two 3 day periods during 4th wk.		
				Nicotinic acid	Quinolinic acid†	N <sup>1</sup> -Me
			<i>gm. per wk.</i>	<i>γ per day</i>	<i>γ per day</i>	<i>γ per day</i>
I	1	None	1.0	4.3 ± 0.03*	6.2 ± 1.6*	7.9 ± 2.2*
	2	0.02 mm (4.08 mg.) L-tryptophan per day intraperitoneally	10.9	11.8 ± 0.6	471 ± 43	243 ± 13
	3	0.02 mm (3.06 mg.) 3-hydroxyanthranilic acid per day intraperitoneally	13.2	12.5 ± 1.0	325 ± 32	141 ± 18
	4	0.02 mm (3.34 mg.) quinolinic acid per day intraperitoneally	10.4	6.0 ± 0.6	1930 ± 250	25.0 ± 4.9
II	1	None	0.75			
	2	0.02 mm quinolinic acid daily as oral supplement	8.5			

\* Standard error.

† Expressed as nicotinic acid.

phan in supporting growth. In Experiment III the levels chosen did not effectively demonstrate the quantitative relationship, but it is evident that 20 mg. per cent of quinolinic acid were slightly more effective than 0.4 mg. per cent of nicotinic acid, or a relative activity ratio of less than 50 on a weight basis. 100 mg. per cent of quinolinic acid were better than 1.0 mg. per cent of nicotinic acid, or a ratio of less than 100.

In Experiment IV quinolinic acid was compared with tryptophan. It was found that 0.25 mm of DL-tryptophan was a more effective supplement than either 0.25 or 0.625 mm of quinolinic acid. These results appear in-

consistent with the postulate that quinolinic acid is an intermediate in the conversion of tryptophan to nicotinic acid. Two possible explanations for this result are evident. Since the ration is limiting in tryptophan, the added growth may be due, in part, to the additional tryptophan, which can be used for purposes other than nicotinic acid synthesis. Presumably quinolinic acid could spare tryptophan only by providing nicotinic acid. The tryptophan may be more effective because of its greater availability at the site of transformation. The rapid excretion of quinolinic acid referred to above may be another factor. Further experimentation is required before the explanation for this non-equivalence of tryptophan and quinolinic acid can be established. There can be no question that quino-

TABLE III

*Relative Activity of Nicotinic Acid, Quinolinic Acid, and Tryptophan in Promoting Growth of Male Rats*

Experiment No.	Group. No.	Supplement to 100 gm. basal ration	Growth for 4 wks. <i>gm. per wk.</i>
III	1	None	0.58
	2	0.4 mg. nicotinic acid	2.6
	3	1.0 " " "	5.7
	4	1.0 " quinolinic "	0.67
	5	5.0 " " "	0.16
	6	20.0 mg. quinolinic acid	3.6
IV	7	100.0 mg. quinolinic acid	7.0
	1	1 mg. nicotinic acid	10.3
	2	51 mg. (0.25 mm) DL-tryptophan	16.1
	3	42 " (0.25 " ) quinolinic acid	7.0
	4	105 " (0.625 " ) " "	9.4

linic acid did exhibit true activity, for the amount of nicotinic acid present in the highest level of quinolinic acid fed would not elicit a growth response.

*Studies with Neurospora*—Of the nicotinamide-requiring mutants of *N. crassa* at least two (mutants 4540 and 3416) fail to respond to any of the known precursors of nicotinic acid. Quinolinic acid has been tested and found inactive for other *nicotinamideless* mutants (7, 8). Since the earlier work (17) with nicotinamide-requiring mutants had shown that the pH of the medium determined the extent to which nicotinic acid could substitute for nicotinamide, presumably because of failure of the dissociated acid to enter the cells, this matter was reinvestigated. Quinolinic acid at concentrations up to 10  $\gamma$  per ml., sterilized separately at pH 7.0 to prevent decarboxylation, failed to support growth of either mutant<sup>1</sup> at pH 3 or 5

<sup>1</sup> Cultures were kindly supplied by Dr. H. K. Mitchell, California Institute of Technology.

in 4 days. Likewise, concentrations of quinolinic acid diamide up to 0.1 mg. per ml. at pH 5 failed to replace nicotinamide for either mutant. However, concentrations of quinolinic acid above 0.05 mg. per ml. supported the growth of mutant 4540. Concentrations as high as 5 mg. per ml. failed to show activity for mutant 3416. Growth of neither mutant in the presence of 0.5  $\gamma$  per ml. of nicotinamide was affected by concentrations of quinolinic acid up to 5 mg. per ml.

The results of one experiment, in which the quinolinic acid was sterilized by filtration to preclude the possibility of slight decarboxylation during autoclaving, are shown in Table IV. It will be noted that the response of mutant 4540 to 500  $\gamma$  of quinolinic acid was greater at the lower pH. That the response of mutant 4540 is not due to nicotinic acid present in the

TABLE IV

*Utilization of Quinolinic Acid by Nicotinic Acid-Requiring Mutants of N. crassa*

Supplement per 10 ml. medium		Weight of mycelium after 5 days growth			
		Mutant 3416		Mutant 4540	
Nicotinic acid	Quinolinic acid	pH 3.3	pH 5.1	pH 3.3	pH 5.1
$\gamma$	$\gamma$	mg.	mg.	mg.	mg.
0	0	0	0	0	0
2	0	16.6	0.6	3.0	0.5
5	0	40.0	44.9	30.9	39.3
10	0	63.3	59.8	40.5	59.1
25	0	39.7	78.5	43.4	62.3
0	500	0	0	71.8	2.4
0	2,500	0	0	71.5	86.9
0	10,000	0	0	77.6	90.1
2	2,500	19.3	4.5	59.9	88.0

quinolinic acid is indicated by the failure of mutant 3416 to respond to high concentrations and by the results of assays of the quinolinic acid used with *L. arabinosus*. 10 mg. of the quinolinic acid contained less than 0.2  $\gamma$  of nicotinic acid as an impurity.

The possible significance of this low activity of quinolinic acid for *N. crassa* 4540 is emphasized by the fact that quinolinic acid was produced in the medium when mutant 3416 was grown in the presence of suboptimum quantities of nicotinamide. This mutant was grown for 6 days in 5 liters of basal medium (18) containing 2.5 mg. of nicotinamide at pH 5.5 in a 20 liter carboy. A stream of filtered air was used to aerate the culture. At the end of 4 days growth, analysis of an aliquot indicated that only a trace of nicotinamide remained, but 92 mg. of nicotinic acid activity were found following acetic acid treatment. After 2 more days the mycelium was removed by filtration and after drying weighed 16.2 gm. The medium contained 158 mg. of quinolinic acid, 37 mg. of which were isolated by the

procedure which has been described for rat urine (12). The active compound exhibited the same behavior as did quinolinic acid in each step of the isolation procedure. It was further identified by melting point, mixed melting point, ultraviolet absorption spectrum, and biological activity following decarboxylation.

#### DISCUSSION

While these results do not prove conclusively that the transformation of tryptophan through 3-hydroxyanthranilic acid to nicotinic acid or its amide proceeds through quinolinic acid, they do strongly suggest this possibility. The fact that tryptophan has been found only approximately 2 per cent as effective as nicotinic acid in promoting the growth of rats receiving 9 per cent casein rations (19) and the rather small extent to which administered tryptophan can be recovered as urinary nicotinic acid and related compounds have led to the view that the over-all conversion of tryptophan to nicotinic acid is very inefficient. The similar low order of activity of 3-hydroxyanthranilic acid (9) would suggest that the limiting reaction in the series from tryptophan to nicotinic acid is between 3-hydroxyanthranilic acid and nicotinic acid. The very rapid conversion of 3-hydroxyanthranilic acid to quinolinic acid by rat liver slices and homogenates (20) and the excretion of the major part of the administered quinolinic acid unchanged would suggest that the limiting reaction in the rat is the decarboxylation of quinolinic acid. It seems possible that the low activity of administered quinolinic acid may be the result of rapid excretion. Its slow formation from 3-hydroxyanthranilic acid within certain cells may permit greater retention and more efficient decarboxylation than when it comes from an exogenous source.

The accumulation of quinolinic acid in the medium of *N. crassa* 3416 and its nicotinic acid activity for mutant 4540 lend support to the hypothesis that quinolinic acid is a true intermediate. On the other hand, quinolinic acid may result from a reaction, such as ring closure, of the true intermediate and the biological activity of quinolinic acid may be a result of the reversal of this reaction.

#### SUMMARY

1. A quantitative method for the determination of quinolinic acid in rat urine, involving decarboxylation to nicotinic acid, has been described.

2. The injection of 1 mm per day of tryptophan or 3-hydroxyanthranilic acid into adult rats receiving a 9 per cent casein ration resulted in approximately a 10-fold increase in the urinary excretion of nicotinic acid, 25- to 100-fold increase in *N*<sup>1</sup>-methylnicotinamide, and 100- to 300-fold increase in quinolinic acid. The urinary levels of all three metabolites decreased toward normal values when the injections were discontinued. When 0.02

mm per day of quinolinic acid was injected into young growing rats, approximately 80 per cent was excreted unchanged. There was some increase in the excretion of nicotinic acid and *N*<sup>1</sup>-methylnicotinamide.

3. Tryptophan, 3-hydroxyanthranilic acid, quinolinic acid, and nicotinic acid were compared with regard to their ability to support the growth of rats receiving a 9 per cent casein-cystine-threonine diet. Intraperitoneally, quinolinic acid was slightly inferior to and 3-hydroxyanthranilic acid was more effective than tryptophan. When the quinolinic acid was incorporated into the ration, it was found one-one hundredth to one-twentieth as active as nicotinic acid and less than half as active as DL-tryptophan.

4. Quinolinic acid was isolated in relatively large amounts from the medium in which mutant 3416 of *Neurospora crassa* had grown with suboptimum levels of nicotinamide. *Neurospora* mutant 4540 utilized quinolinic acid as a source of nicotinic acid only when the concentration in the medium was approximately 0.05 mg. per ml.

5. The question of the possible intermediary rôle of quinolinic acid in the conversion of tryptophan to nicotinic acid is discussed.

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# QUINOLINIC ACID METABOLISM

## III. FORMATION FROM 3-HYDROXYANTHRANILIC ACID BY RAT LIVER PREPARATIONS

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The administration of tryptophan to the rat is followed promptly by marked increases in the urinary excretion of quinolinic acid (1, 2), *N*<sup>1</sup>-methylnicotinamide, and nicotinic acid (2-5). Experiments in which the intestinal tracts have been removed surgically indicate that the conversion of tryptophan to these metabolites occurs largely in the tissues and is not influenced by the intestinal microflora (6, 7).

Hurt *et al.* (8) reported studies *in vitro* which showed that the incubation of tryptophan with liver slices increased the nicotinic acid content as determined by analysis with *Lactobacillus arabinosus* following autoclaving for 1 hour with 1 *N* sulfuric acid. Schweigert (9) demonstrated the conversion of 3-hydroxyanthranilic acid by liver slices to a compound which becomes active for *L. arabinosus* when autoclaved with 1 *N* sulfuric acid. The results reported here show that the active substance formed under these conditions is primarily, and probably entirely quinolinic acid, which was partially decarboxylated by the hydrolysis procedure employed by the above workers. No increase in the nicotinic acid or quinolinic acid content of liver slices could be demonstrated when they were incubated with low concentrations of tryptophan or kynurenine.

### EXPERIMENTAL AND RESULTS

**Methods**—Standard procedures were used for preparing and incubating preparations of rat liver. The animals were exsanguinated and the liver rapidly removed, chilled, and sliced or homogenized with a Potter-Elvehjem homogenizer in cold Krebs-Ringer phosphate buffer. Substrates were added to 3 ml. of buffer, at pH 7.2, containing slices or homogenate at 37° in Warburg flasks or test-tubes. After being shaken slowly for definite periods of time, exposed to the atmosphere, the contents were removed, placed in a boiling water bath for 3 minutes, homogenized, and diluted to 10 ml. with distilled water. Aliquots were removed for determination of nicotinic acid and quinolinic acid (10).

**Isolation of Product**—The properties of the substance formed when liver slices are incubated with 3-hydroxyanthranilic acid suggested that it was

quinolinic acid. In particular, the slow formation of nicotinic acid during autoclaving with 1 N sulfuric acid and the rapid reaction on heating with acetic acid corresponded well with the behavior of quinolinic acid. That the product is quinolinic acid was proved by the isolation of this compound from an incubation mixture.

100 mg. of 3-hydroxyanthranilic acid were incubated for 25 minutes at 37° with 10.4 gm. of homogenized rat liver in 200 ml. of Krebs-Ringer phosphate buffer in a 2 liter Erlenmeyer flask with intermittent shaking. An additional 4.6 gm. of freshly homogenized liver were then added and the incubation continued for 15 minutes. The mixture was heated on a steam bath for 10 minutes, filtered, and the filtrate and washings were diluted to 250 ml. The solution contained 2.0 mg. of nicotinic acid and 48.5 mg. of quinolinic acid. After dilution to 1 liter, the solution was adjusted to pH 1.2 with concentrated hydrochloric acid and stirred with 25 gm. of norit for 1 hour. The quinolinic acid was eluted from the norit with 1 liter of 0.1 N ammonium hydroxide and the filtrate was concentrated to dryness. The residue weighed 780 mg.; it contained 38.3 mg. of quinolinic acid but no nicotinic acid. The white solid was dissolved in 25 ml. of methanol adjusted to pH 2 and chromatographed on 50 gm. of alumina in an 11 cm. column (2). After being washed with 300 ml. of methanol and 400 ml. of distilled water, the column was developed with approximately 0.03 N ammonium hydroxide. The fraction coming from the column from pH 8.7 to 9.6 gave a slight test with ferrous sulfate and contained 29.5 mg. of quinolinic acid. This corresponded to 49.5 per cent pure diammonium salt. After conversion to the free acid by addition of hydrochloric acid to pH 2, the product was dried *in vacuo* and crystallized from 40 per cent acetic acid. After recrystallization from the same solvent, approximately 4 mg. of crystalline quinolinic acid were obtained. On the micro block, the crystals lost their luster and began to sublime at 165–170°; at 187–188° they melted and promptly formed new crystals which melted at 225–230° with sublimation when heated rapidly. There was no alteration of this behavior when the substance was mixed with authentic quinolinic acid. The ultraviolet absorption spectrum agreed closely with that of quinolinic acid. Autoclaving with glacial acetic acid resulted in the theoretical quantity of nicotinic acid. These findings are considered proof that the product formed by the action of liver preparations on 3-hydroxyanthranilic acid is quinolinic acid.

*Studies with Liver Slices and Homogenates*—The results of an experiment designed to determine the effect of various reagents on the release or formation of nicotinic acid from incubated liver slices are summarized in Table I. Following incubation under the conditions indicated above, aliquots were removed and treated in various ways prior to the determina-



tion of nicotinic acid in each. Strong alkali had no effect on the nicotinic acid content. Likewise in the control flasks (Nos. 1 and 2) to which no substrate was added, neither acetic acid nor sulfuric acid treatment affected the values significantly. Incubation of the slices with 3-hydroxyanthranilic acid or quinolinic acid did not influence the "free" nicotinic acid content. That quinolinic acid or some similar compound was formed in Flask 3 is evident from the results of analysis following autoclaving with acetic acid. In Flask 4, more than 80 per cent of the quinolinic acid added was recovered after incubation for 3 hours. The results with the sulfuric acid-treated aliquots indicate that the slight conversion of 3-hydroxyanthranilic acid to nicotinic acid, reported by Schweigert (9), was the result of partial decarboxylation of quinolinic acid by the procedure employed for releasing the nicotinic acid. When samples were autoclaved for 30 minutes

TABLE I

*Nicotinic Acid and Quinolinic Acid Content of Rat Liver Slices Incubated with 3-Hydroxyanthranilic Acid*

Flask No.	Substrate added and incubation time	Fresh weight of slices	Nicotinic acid per flask			
			No treatment	1 N NaOH autoclaved 15 min.	Acetic acid autoclaved 2 hrs.	1 N H <sub>2</sub> SO <sub>4</sub> autoclaved 30 min.
		mg.	$\gamma$	$\gamma$	$\gamma$	$\gamma$
1	None, Boiled, No incubation	358	44.8	44.0	51.5	44.5
2	None, incubated 3 hrs.	329	42.0	42.3	45.5	44.7
3	2 $\mu$ M 3-hydroxyanthranilic acid incubated 3 hrs.	349	49.2	47.5	222	58.2
4	2 $\mu$ M quinolinic acid, incubated 3 hrs.	386	53.7	51.5	257	72.0

with 1 N sulfuric acid, the results were in good agreement with those he has published.<sup>1</sup>

When L-tryptophan and kynurenine were incubated with the liver slices, no increase in their nicotinic acid or quinolinic acid content resulted (Table II), while, in the same experiment, 50 per cent of the 3-hydroxyanthranilic acid was converted to quinolinic acid and 95 per cent of the quinolinic acid added as a substrate was recovered unchanged. There was no evidence that nicotinic acid was formed during incubation with any of the substrates. In Flasks 1, 2, 3, and 7, some increase in the nicotinic acid values resulted from the acetic acid treatment, suggesting that the fresh liver contained a small amount of quinolinic acid.

<sup>1</sup> In a private communication Dr. Schweigert has stated that in more recent experiments acetic acid was much more effective than sulfuric acid in forming the nicotinic acid-like substance (or substances) he has measured.

TABLE II

*Nicotinic Acid and Quinolinic Acid Content of Rat Liver Slices As Affected by Incubation for 3 Hours with Various Substrates*

Flask No.	Substrate added	Fresh weight of slices	Nicotinic acid per flask		
			No. treatment	Acetic acid autoclaved 2 hrs.	Increase from acetic acid treatment*
		mg.	$\gamma$	$\gamma$	$\gamma$
1	None	298	38.0	42.5	4.5
2	2 $\mu$ M L-tryptophan	287	36.7	43.3	6.6
3	2 " kynurenine	382	49.5	54.5	5.0
4	2 " 3-hydroxyanthranilic acid	453	57.8	182	124 (51%)
5	2 " quinolinic acid	366	45.5	279	233 (95%)
6	2 " 3 - hydroxyanthranilic acid (no liver)	0	0	0	0
7	None, boiled at 0 time	362	38.8	51.3	12.5

\* To convert to quinolinic acid multiply by 1.36.

TABLE III

*Effect of Time and Weight of Liver on Conversion of 3-Hydroxyanthranilic Acid to Quinolinic Acid*

Experiment No.	Flask No.	Fresh weight of liver used	Substrate	Incubation period	Type of preparation	Nicotinic acid per flask	
						No treatment	Autoclaved 2 hrs. with acetic acid
		mg.	$\mu$ M	min.		$\gamma$	$\gamma$
1	1	306	2	Boiled 0 time	Slices	40.9	49.5
	2	286	2	6	"	35.0	170
	3	302	2	11	"	40.8	158
	4	317	2	30	"	40.2	159
	5	312	2	60	"	43.2	164
	6	300	2	60	Homogenate	42.5	140
2	1	300	0	0	"	42.5	53.0
	2	300	0	60	"	51.5	56.3
	3	300	2	5	"	49.8	198
	4	300	2	30	"	50.8	184
	5	100	2	5	"	18.1	160
	6	100	2	30	"	17.0	226
	7	50	2	5	"	9.3	120
	8	50	2	60	"	8.8	252
	9	300	2*	30	"	52.0	267
	10	300	2*	60	"	52.0	265

\* Quinolinic acid.

Preliminary studies of the reaction rate and a comparison of results obtained with slices and homogenates are reported in Table III. In Experiment 1, in which approximately 300 mg., wet weight, of liver and 2  $\mu$ M of substrate were used, 6 minutes of incubation resulted in as much conversion as longer periods. The homogenate was nearly as active as were slices. Experiment 2 was performed with the homogenate of the liver of a normal, adult rat. With 300 mg. of tissue, maximum conversion resulted in 5 minutes; with 100 mg. of tissue, 30 minutes gave higher values than did 5 minutes; and with 50 mg. of liver, 60 minutes of incubation resulted in more oxidation of the substrate than did 5 minutes. That a very active enzyme system is involved is indicated by the fact that 50 mg. of tissue converted nearly 1  $\mu$ M of 3-hydroxyanthranilic acid to quinolinic acid in 5 minutes. As with slices, most of the quinolinic acid added was recovered unchanged. There appeared to be somewhat more "free" nicotinic acid present, especially following the longer periods of incubation, but from these data it is not possible to determine whether it is formed from quinolinic acid or is released from other compounds during incubation.

#### DISCUSSION

These experiments *in vitro* provide substantiating evidence that 3-hydroxyanthranilic acid, an intermediate in the synthesis of nicotinic acid by *Neurospora*, is converted to quinolinic acid by animal tissues, and further suggest that quinolinic acid is an intermediate in the established conversion of tryptophan to nicotinic acid. Some of the findings reported here are at variance with those of Hurt *et al.* (8), since little, if any, nicotinic acid was formed during incubation of liver preparations with tryptophan, kynurenine, 3-hydroxyanthranilic acid, or quinolinic acid. It seems likely that these workers were determining a small portion of the quinolinic acid which was decarboxylated by their hydrolytic treatment. It is not evident why they observed increased synthesis of nicotinic acid-like compounds when tryptophan was added, whereas, under the conditions of our experiments, no measurable increase occurred. Under very similar experimental conditions, Schweigert (9) also observed no conversion of tryptophan to nicotinic acid derivatives.

#### SUMMARY

1. Quinolinic acid is formed when 3-hydroxyanthranilic acid is incubated with rat liver slices or homogenates. This compound was isolated and identified by melting point, ultraviolet absorption spectrum, and biological activity following decarboxylation to nicotinic acid.

2. Incubation of liver slices with tryptophan, kynurenine, 3-hydroxyanthranilic acid, or quinolinic acid did not increase their "free" nicotinic

acid content significantly. 3-Hydroxyanthranilic acid was the only substrate tested which gave a measurable increase in the quinolinic acid content of liver slices. Rat liver contains small amounts of quinolinic acid or other substances which become active for *Lactobacillus arabinosus* when autoclaved for 2 hours with glacial acetic acid.

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# PHOSPHORIC ESTERS OF BIOLOGICAL IMPORTANCE

## II. THE SYNTHESIS OF GLUCOSE-6-PHOSPHATE FROM 1,2-ISOPROPYLIDENE-5,6-ANHYDRO-D-GLUCOFURANOSE\*

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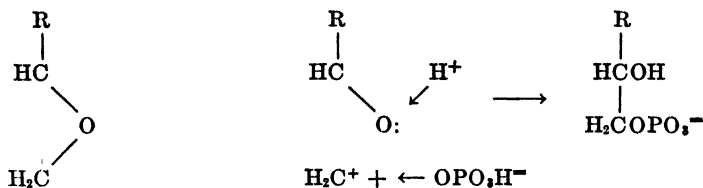
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Ohle and coworkers (2-7), in their investigations of reactions of 1,2-isopropylidene-5,6-anhydro-D-glucofuranose, found that a wide variety of acid and alkaline reagents would cleave the ethylene oxide ring with the attachment of the anionoid groups to carbon atom 6. Among others, the 6-amino, 6-sulfhydryl, 6-halo, and 6-alkoxy derivatives were prepared. Their results suggested the possibility that the anhydro ring could also be cleaved by inorganic phosphate with the formation of the 6-phosphate ester. If such a synthesis could be achieved, it would lend itself exceptionally well to syntheses with radioactive phosphorus. The direct esterification would eliminate the necessity of converting the radioactive orthophosphate to any of the variety of acid chlorides used currently for the synthesis of phosphoric esters.

In our investigations of the reaction, 1,2-isopropylidene-5,6-anhydro-D-glucofuranose was heated in an aqueous solution of various orthophosphate compounds; namely, phosphoric acid, monobasic phosphate, dibasic phosphate, and tribasic phosphate. No esterification of inorganic phosphate into organic phosphate was observed with any of these reagents with the notable exception of dibasic phosphate. The failure of phosphoric acid and monobasic phosphate to esterify can be explained on the basis that, in an aqueous acid solution, scission of the anhydro ring usually results in the formation of free sugars without the attachment of any groups (8). The reason that dibasic phosphate cleaves the ring with the attachment of a phosphate group, while tribasic phosphate does not, is not so readily explained. It is possible that, because the tribasic salt in solution has a high pH, the anhydro ring is cleaved without the attachment of a phosphate group as the result of a high concentration of hydroxyl ions. Dibasic phosphate in solution is only slightly alkaline, with a much lower concentration of hydroxyl ions; thus there are relatively more phosphate anions to attack the carbonium ion formed at carbon atom 6. A possible mechanism of the reaction (9) is shown in the accompanying diagram. Another possi-

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. For Paper I of this series see Lardy and Fischer (1). Supported in part by a grant from the United States Public Health Service (RG 313).

bility is that complex formation between the anhydro sugar and orthophosphate occurs prior to cleavage of the anhydro ring.



The phosphorylating reaction was carried out by refluxing a solution containing equimolar concentrations of reactants, or an excess of phosphate, for 24 and 48 hours to determine the conditions for maximum uptake of inorganic phosphorus. The results are shown in Table I.

TABLE I  
*Influence of Time and Amount of Phosphate on Yield of Glucose-6-phosphate*

$\text{K}_2\text{HPO}_4$ per mole anhydro sugar	Time of refluxing	Per cent yield*
<i>moles</i>	<i>hrs.</i>	
1.0	24	40.1
1.0	48	47.8
1.8	24	51.0
1.8	48	51.2

\* Calculated on the basis of orthophosphate disappearance.

It was found that 1.8 moles of  $\text{K}_2\text{HPO}_4$  per mole of the anhydroglucose derivative gave the best conditions for the reaction, since the esterification was practically complete at the end of 24 hours in the presence of an excess of phosphate. Heating the reactants under pressure at 120° did not increase the yield. By further increasing the molar concentration of orthophosphate a greater percentage of the anhydro compound was esterified, but the yield of glucose-6-phosphate isolated was not appreciably increased because of losses due to occlusion in the larger quantities of barium phosphate. Increasing the relative quantity of the anhydro compound results in more complete esterification of the phosphate (10), but is not practical in large scale preparations of glucose-6-phosphate since the preparation of the starting material does not proceed with good yields (11). The isopropylidene group was removed by heating in dilute HBr and the phosphate ester was isolated as the barium salt.

#### EXPERIMENTAL

1,2-Isopropylidene-5,6-anhydro-D-glucofuranose was prepared<sup>1</sup> according to Ohle and Vargha (11). 1.9 gm. of the anhydro sugar derivative

<sup>1</sup> We are indebted to Dr. Harry L. Willard of the Corn Products Refining Company for generous supplies of 1,2-isopropylidene-D-glucofuranose.

(0.01 mole) were added to 3.1 gm. of  $K_2HPO_4$  (0.018 mole) dissolved in 50 cc. of water in a round bottom flask. After refluxing for 24 hours the reaction mixture was cooled, HBr was added to about pH 1, and the solution was heated on the steam bath for 20 to 30 minutes to split off the isopropylidene group (positive Fehling's test). The mixture was cooled, and pulverized  $Ba(OH)_2$  was added to neutrality. The barium phosphate was centrifuged, washed twice with 25 cc. portions of distilled water, and the supernatant solutions were combined. The isolation and drying of the barium salt of glucose-6-phosphate were carried out according to the procedure of Lardy and Fischer (1). 4 volumes of 95 per cent ethanol were added, and, after the precipitate had settled, the supernatant liquor was decanted. The barium salt was centrifuged and washed in succession with 95 per cent ethanol, absolute ethanol, 75 per cent ethanol-25 per cent ether, 25 per cent ethanol-75 per cent ether, and finally with dry ether.

After being dried in air, the barium glucose-6-phosphate was dissolved by extraction with three successive portions of distilled water. To the clear filtrate, 4 volumes of ethanol were added and the product was separated and dried as above. The final drying was *in vacuo* over  $P_2O_5$ .

The yield was 1.52 gm. (41 per cent of the theory) of the barium salt, which was free of inorganic phosphate and was 90 per cent pure on the basis of its organic phosphorus content. Its rotation (purity based on P content) was  $[\alpha]_D^{24} = +16.3^\circ$ .

The dried salt probably retains water. The analyses correspond with a salt containing 2.5 moles of  $H_2O$ .

$C_6H_{11}O_6PBa$ (395.5).	Calculated.	Ba 34.7, P 7.83
$C_6H_{11}O_6PBa \cdot 2.5H_2O$ (440.5).	"	" 31.1, " 7.04
	Found.	" 31.1, " 7.05

Barium was determined by the colorimetric procedure of Frediani and Babler (12), adapted to the Evelyn colorimeter.

The biological activity of glucose-6-phosphate prepared by this method was practically identical with that of the same compound prepared from 1,2,3,4-tetraacetyl- $\beta$ -D-glucopyranose (1) when used as a substrate for phosphohexokinase (13).

#### SUMMARY

A new procedure is described for the synthesis of glucose-6-phosphate. The ethylene oxide ring of 1,2-isopropylidene-5,6-anhydro-D-glucofuranose was cleaved by dibasic phosphate in water at  $100^\circ$  with esterification of phosphate to the terminal carbon atom. The over-all yield, starting from glucose, is not as good as that realized in the method described by Lardy and Fischer. The procedure is better adapted, however, to syntheses involving radioactive phosphorus, since the latter can be used directly in the orthophosphate form.

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# PHOSPHORIC ESTERS OF BIOLOGICAL IMPORTANCE

## III. THE SYNTHESIS OF PROPANEDIOL PHOSPHATE\*

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(Received for publication, July 15, 1949)

In a recent publication Lindberg (1) reported the occurrence in brain of a new ester, propanediol phosphate, to the extent of 5 per cent of the acid-soluble organic phosphorus. Appreciable quantities were found also in rat liver and kidney and the eggs of certain marine forms. LePage (2) has isolated this ester from rat carcinoma. The interesting biological properties of the compound (1) have prompted us to develop a convenient method for its synthesis.

Lindberg's synthesis of propanediol phosphate from 1,2-propanediol and  $\text{POCl}_3$  resulted in a mixture of the racemic forms of both  $\alpha$ - and  $\beta$ -phosphates, which would undoubtedly have much less activity than the natural ester. The method also gave very low yields, which made it difficult to obtain the ester in sufficient quantities for further study of its biological significance.

In previous studies in this laboratory (3), a new method for the synthesis of glucose-6-phosphate was developed. Dipotassium phosphate was incorporated directly into the ester by heating 5,6-anhydroisopropylidene glucose with aqueous  $\text{K}_2\text{HPO}_4$ . Since the position of attachment of the phosphate group, and also of a wide variety of other entering groups on cleavage of the anhydro ring (4), is on the terminal carbon atom, it seemed reasonable to assume that similar treatment of commercially available propylene oxide would yield the  $\alpha$ -phosphoric ester of 1,2-propanediol.

The reactions involved in the new synthesis and the isolation of the lead salt are shown in the accompanying diagram.

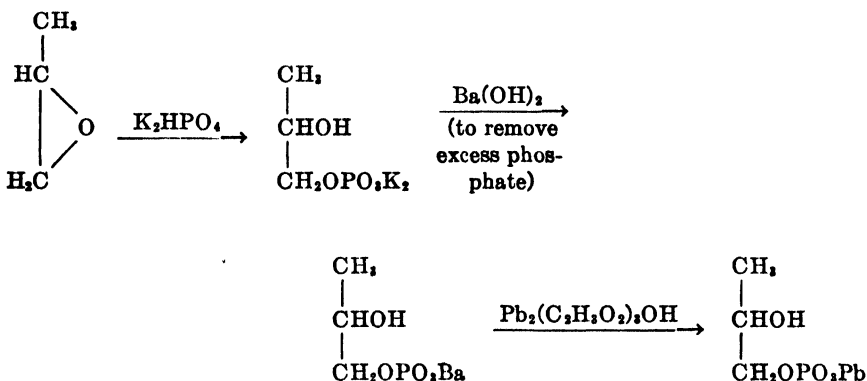
The synthesis is especially convenient for the preparation of propanediol phosphate containing  $\text{P}^{32}$ , since  $\text{K}_2\text{HP}^{32}\text{O}_4$  may be employed directly. Under prescribed conditions complete conversion of inorganic orthophosphate to propanediol phosphate can be achieved.

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## EXPERIMENTAL

1 ml. of propylene oxide<sup>1</sup> was added to a solution of 3.3 gm. of  $K_2HPO_4 \cdot 3H_2O$  (1:1 mole) in 24 ml. of water in a pressure bottle apparatus and placed on the steam bath for 12 hours. Aliquots for analysis of phosphorus uptake (5) were taken before and after heating the reactants. After 12 hours, 62 per cent of the phosphate had been esterified. Prolonging the period of heating did not increase the amount of phosphate fixed. The reaction mixture was removed from the steam bath, cooled, and solid barium acetate was added to remove the unchanged phosphate. A little barium hydroxide solution was added to keep the mixture slightly alkaline.

The barium phosphate was centrifuged, washed twice with a small amount of distilled water, and the supernatant solutions were combined. A few drops of barium hydroxide solution were added to test the complete-



ness of precipitation. The water-soluble barium salt of the phosphoric ester was converted to the sparingly soluble lead salt according to the method of LePage (2). The clear solution was brought to pH 7.8 with dilute  $HNO_3$  and a filtered saturated solution of basic lead acetate was added. A fine white precipitate formed which was removed by centrifugation, washed, and dried over  $P_2O_5$  *in vacuo*; yield, 2.8 gm. (54 per cent of theoretical) of the lead salt.

The compound was characterized as follows:

	Theoretical per cent	Found per cent
Phosphorus.....	8.58	8.36
Lead.....	57.3	56.5
	Synthetic ester	Isolated ester (1)
P hydrolyzed in 3 hrs. in 1 N HCl at 99°.....	2.5	2.1
P hydrolyzed in 30 min. in 0.5 N NaOH at 99°.....	0	0

<sup>1</sup> Generously supplied by The Dow Chemical Company, Midland, Michigan.

Further studies on the conditions of the reaction were carried out in order to obtain a greater increase in phosphorus uptake for the synthesis of propanediol phosphate with radioactive phosphorus. Increasing the concentration of propylene oxide was tried in the hope that more of the inorganic phosphate could be esterified. The results of studies with various molar ratios of propylene oxide to  $K_2HPO_4$  are shown in Fig. 1. All reactions were carried out in duplicate under pressure in a similar concentration of phosphate in water for the same period of heating (12 hours).

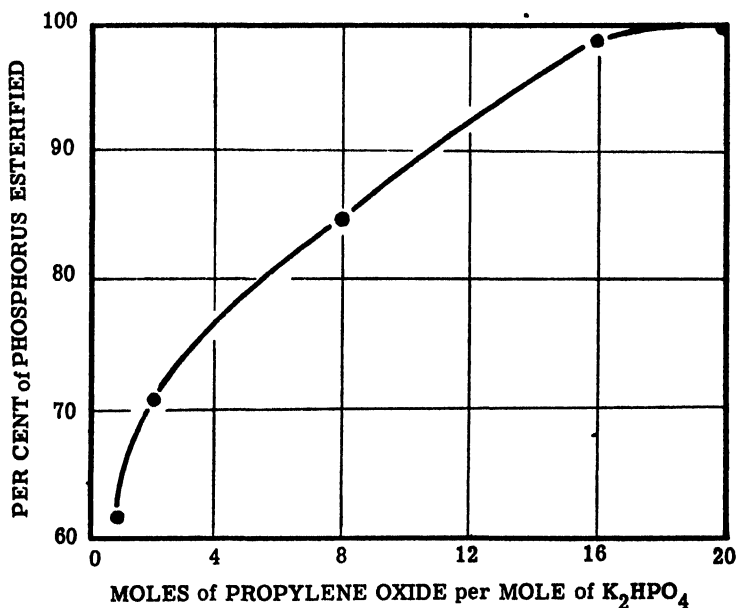


Fig. 1. Influence of varying ratios of propylene oxide to phosphate on the esterification of phosphate.

By having a large excess of propylene oxide (20 moles to 1), in the reaction mixture, it is possible to incorporate 100 per cent of the inorganic phosphorus into organic phosphoric ester. Thus, in one reaction step, radioactive phosphorus can be directly converted quantitatively into 1,2-propanediol- $\alpha$ -phosphate. The product formed when the molar concentration of propylene oxide was increased to 20:1 was isolated as the lead salt and analyzed chemically to determine whether it was the same compound as that produced when the molar ratio of the reactants was 1:1. The procedure can be adapted as follows to the synthesis of propanediol phosphate containing  $P^{32}$ .

2 ml. of propylene oxide were added to a solution of 0.33 gm. of  $K_2HPO_4$ .

$3\text{H}_2\text{O}$  dissolved in 5 ml. of distilled water in a pressure bottle apparatus and placed on the steam bath for 12 hours. Since the analyses showed a complete disappearance of orthophosphate from the reaction mixture, no addition of barium acetate was necessary to remove unchanged phosphate. After the reaction mixture had been cooled and the pH adjusted to 7.8, 2 ml. of a filtered saturated solution of basic lead acetate were added. The lead salt of propanediol phosphate was removed by centrifugation, washed with a small quantity of distilled water, and dried over  $\text{P}_2\text{O}_5$  *in vacuo*. The yield was 0.42 gm. (87 per cent of the theoretical).

$\text{C}_3\text{H}_7\text{O}_4\text{PPb}$  (361.3). Calculated, P 8.58, Pb 57.3; found, P 8.45, Pb 56.9

After hydrolysis for 3 hours in 1 N HCl at  $99^\circ$ , 2.3 per cent of the phosphorus was liberated, indicating that the compound is the same as that described in the previous data.

#### SUMMARY

A new one step procedure is described for the synthesis of DL-1,2-propanediol- $\alpha$ -phosphate from commercially available propylene oxide and  $\text{K}_2\text{HPO}_4$ . When an aqueous solution of the reactants is heated under pressure, the ethylene oxide ring is cleaved, with the attachment of a phosphate group.

When the molar ratio of propylene oxide to  $\text{K}_2\text{HPO}_4$  was increased to 20:1, quantitative esterification of the phosphate occurred. This procedure is especially adaptable to the synthesis of propanediol phosphate from radioactive phosphorus.

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## PHOSPHORIC ESTERS OF BIOLOGICAL IMPORTANCE

### IV. THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF D-TAGATOSE-6-PHOSPHATE\*

By EZRA L. TOTTON† AND HENRY A. LARDY

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(Received for publication, August 4, 1949)

A metabolic pathway by which glucose, fructose, and mannose are metabolized in mammalian tissue has been elucidated. This is not the case, however, for galactose, a sugar whose metabolic significance is evinced by the fact that it provides half of the dietary supply of carbohydrate for most young mammals and is usually present in the diet of man throughout his life.

Galactose is converted to galactose-1-phosphate in animal and yeast cells, as shown by the work of Kosterlitz (1) and Trucco *et al.* (2). Galactose-1-phosphate is converted into phosphorylated derivatives, presumably of glucose and fructose, as demonstrated by Grant (3) and Caputto *et al.* (4), but little is known about the mechanism of this conversion.

One of the earliest theories advanced for the mechanism of this conversion was that inversion took place at carbon atom 4 of galactose, possibly by phosphorylation and subsequent formation of anhydro sugar. Trucco *et al.* (2, 4) have been the most recent proponents of this theory. However, no investigations yet reported give any definite knowledge of the pathway of this conversion, and Peat (5) has recently questioned the likelihood of its occurrence.

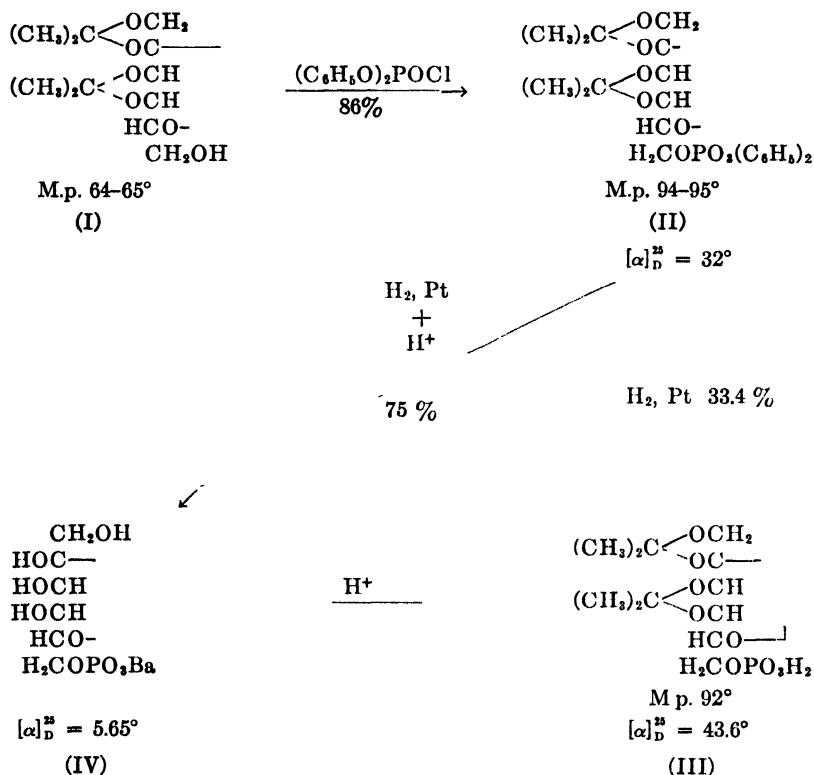
We have undertaken an investigation of the metabolism of D-tagatose-6-phosphate. Tagatose bears the same relationship to galactose as fructose does to glucose, and it is well known that fructose-6-phosphate is an intermediate in glucose metabolism. We wished especially to determine whether tagatose-6-phosphate might be enzymatically phosphorylated to a 1,6-diphosphate, as is fructose-6-phosphate. For, if such a diphosphate were to be cleaved by an aldolase, the same two trioses, dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate, would be formed as from fructose diphosphate. Such, indeed, has now been found to be the case

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and it is tempting to speculate that it is at the triose phosphate stage that galactose metabolism joins a common pathway with the other metabolizable hexoses. Although tagatose-6-phosphate is metabolized by tissue extracts, *it has yet to be determined whether this compound is an intermediate in galactose metabolism.*

D-Tagatose-6-phosphate was synthesized by a procedure outlined in the accompanying diagram. The starting material, D-tagatose (6), was con-



verted to 1,2,3,4-diisopropylidene-D-tagatose (7) and the latter was phosphorylated with diphenyl chlorophosphonate (8). The crystalline 1,2,3,4-diisopropylidene-D-tagatofuranose-6-diphenyl phosphate (II) was converted to 1,2,3,4-diisopropylidene-D-tagatofuranose-6-phosphoric acid (III) by reductive cleavage of the phenyl groups with hydrogen in the presence of Adams' catalyst (9). This free acid was crystallized from pentane. For preparative purposes III is not isolated but is converted directly to tagatose-6-phosphate. The isopropylidene groups were removed from III by acid hydrolysis (a water solution of III was sufficiently acid) and the D-tagatose-6-phosphate was isolated as the barium salt (IV).

For biological experimentation, the barium salt of D-tagatose-6-phosphate was converted in solution to the sodium or potassium salt. D-Tagatose-6-phosphate was found to be phosphorylated by a dialyzed, lyophilized extract of beef brain at approximately the same rate as was glucose-6-phosphate. Comparative rates of phosphorylation are shown in Fig. 1. The products of enzymatic phosphorylation were not characterized, but pre-

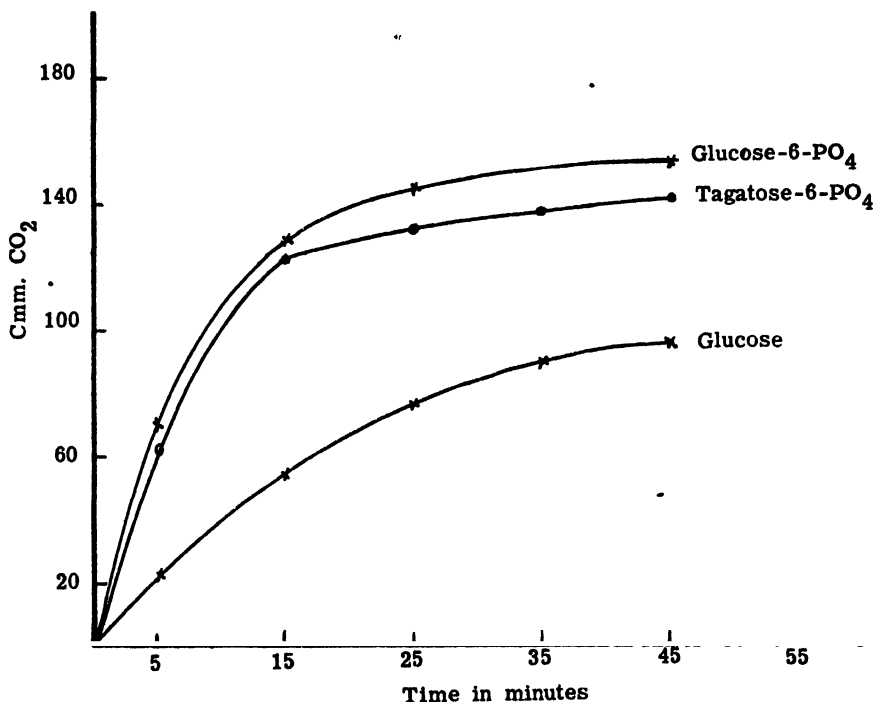


FIG. 1. Phosphorylation of tagatose-6-phosphate, glucose-6-phosphate, and glucose by lyophilized, dialyzed beef brain extract. Adenosine triphosphate 0.0024 M;  $Mg^{++}$  0.008 M; hexose or hexose monophosphates 0.01 M; 6 mg. of brain preparation per flask; final volume 3 ml.;  $T = 37^\circ$ . For details see Weibelhaus and Lardy (12).

sumably a tagatose diphosphate is formed. The brain preparation contains aldolase and an appreciable quantity of triose phosphate accumulates in the reaction mixture. Preliminary data indicate that the triose phosphate arises from a barium-insoluble (hexose diphosphate?) ester. Studies on the metabolism of the tagatose phosphates and their possible rôle in galactose metabolism are in progress.

#### EXPERIMENTAL

*1,2,3,4-Diisopropylidene-D-Tagatose-6-Diphenyl Phosphate (II)*—To 1.5 gm. (0.0057 mole) of 1,2,3,4-diisopropylidene-D-tagatose (I), in 14 ml.

of cold dry pyridine, 1.5 ml. (0.007 mole) of diphenyl chlorophosphonate were added dropwise. After the reaction mixture had remained at 0° for 30 minutes, it was placed in the cold room at 5° for 24 hours. The reaction mixture was poured onto 200 ml. of finely cracked ice while being rapidly stirred. The stirring was continued for 30 minutes and the mixture extracted with 125 ml. of chloroform. The chloroform solution was washed first with 25 ml. of dilute HCl and four times with a total of 100 ml. of distilled water. It was dried over  $\text{Na}_2\text{SO}_4$  (anhydrous) and concentrated under reduced pressure to a dry sirup. The sirup was taken up in 200 ml. of absolute ethanol; distilled water was added to turbidity, and, after scratching, the mixture was allowed to remain at 5° for 24 hours. The product crystallized in long silky needles. After two such recrystallizations, the product weighed 2.5 gm. (86 per cent of theory); m.p. 94–95°;  $[\alpha]_D^{25} = 32^\circ$  ( $c = 2$  in chloroform). Further recrystallization did not change the melting point or the rotation.

*Analysis*— $\text{C}_{24}\text{H}_{38}\text{O}_8\text{P}$  (492.5). Calculated. C 58.51, H 5.93, P 6.28  
 Found. " 58.03, " 6.12, " 6.21  
 " 58.34, " 6.16, " 6.21

*1,2,3,4-Diisopropylidene-D-Tagatose-6-Phosphoric Acid (III)*—A solution of 1.3 gm. (0.0026 mole) of 1,2,3,4-diisopropylidene-D-tagatose-6-diphenyl phosphate in 25 ml. of absolute ethanol was shaken with 0.12 gm. of platinum catalyst (9) and hydrogen at a pressure slightly greater than atmospheric. The reaction stopped after the theoretical amount of hydrogen had been consumed (8 moles). The reaction was complete in 5 hours. After removal of the catalyst by centrifugation, the alcoholic solution was concentrated to a sirup under reduced pressure. The product was obtained in crystalline form from 100 ml. of pentane. After remaining at 5° for 24 hours, the crystals were collected, washed with pentane, and dried over paraffin and calcium chloride; weight 0.300 gm. (33.4 per cent); m.p. 92°;  $[\alpha]_D^{25} = 43.6^\circ$  ( $c = 0.5$  in absolute ethanol).

*Analysis*— $\text{C}_{13}\text{H}_{21}\text{O}_8\text{P}$  (340.3). Calculated. C 42.33, H 6.22, P 9.11  
 Found. " 42.71, " 6.32, " 9.17  
 " 9.17

*Barium-D-Tagatose-6-Phosphate (IV)*—1,2,3,4-Diisopropylidene-D-tagatose-6-diphenyl phosphate (II) (2.5 gm.) was cleaved by reduction with hydrogen and platinum as above. The sirup was crystallized from pentane, and the pentane decanted but no further attempt was made to purify the product. The crystal sludge gave a negative Fehling's test. 15 ml. of distilled water were added to the crystals and the solution warmed on a steam bath for 3 minutes. The solution now gave a strongly positive Fehling's test. Barium hydroxide solution ( $\text{CO}_2$ -free) was added



dropwise until the pH reached 10.2 and, after filtering, the solution (60 ml.) was poured into 240 ml. of 95 per cent ethanol. The mixture was placed in the cold room at 5° for 24 hours. The mother liquor was decanted, and the white amorphous precipitate was collected on the centrifuge. It was washed once with absolute ethanol and once with each of the following absolute ethanol-ether mixtures, 80:20, 50:50, 20:80, and twice with anhydrous ethyl ether. The dried product weighed 1.5 gm. (75 per cent),  $[\alpha]_D^{25} = 5.65^\circ$  ( $c = 1.06$  in water).

*Analysis*<sup>1</sup>— $C_6H_{11}O_6P\text{Ba}$  (395.5). Calculated. C 18.22, H 2.81, P 7.83  
Found. " 17.58, " 3.11, " 7.83  
" 7.84

*Biological Activity*—The rate of phosphorylation of D-tagatose-6-phosphate by adenosine triphosphate was measured by the manometric technique of Colowick and Kalckar (10), with the dialyzed lyophilized beef brain preparation (12) as a source of phosphohexokinase. The curves in Fig. 1 show the comparative results of enzymatic phosphorylation of glucose, D-tagatose-6-phosphate, and D-glucose-6-phosphate (11). D-Tagatose-6-phosphate was phosphorylated at approximately the same rate as glucose-6-phosphate, while glucose was phosphorylated more slowly, as was expected (12). In similar experiments in which fructose-6-phosphate was also used as a substrate the reaction mixtures were analyzed for triose phosphate by treatment with 1 N NaOH at room temperature for 20 minutes. With brain preparations, as much triose phosphate was formed per mole of tagatose-6-phosphate phosphorylated as was formed from fructose-6-phosphate following its conversion to fructose-1,6-diphosphate. When extracts of acetone-powdered rat liver tumor were used as a source of phosphohexokinase and aldolase, triose phosphate was formed much more slowly when tagatose-6-phosphate was the substrate than when glucose-6-phosphate or fructose-6-phosphate was present. D-Tagatose (6) was not phosphorylated by brain hexokinase.

#### SUMMARY

A method for the synthesis of D-tagatose-6-phosphate is outlined. 1,2,3,4-Diisopropylidene-D-tagatose was phosphorylated with diphenyl chlorophosphonate to give 1,2,3,4-diisopropylidene-D-tagatose-6-diphenyl phosphate, which was cleaved with hydrogen and platinum to give 1,2,3,4-diisopropylidene-D-tagatose-6-phosphoric acid. The isopropylidene groups were removed from the diisopropylidene phosphoric acid derivative by heating it in an aqueous solution, and the D-tagatose-6-phosphate was isolated as the barium salt.

<sup>1</sup> The usual difficulties were experienced in obtaining quantitative yields of  $\text{CO}_2$  during combustion of the barium salt.

The biological activity of the sodium salt of D-tagatose-6-phosphate was determined with beef brain extract containing phosphohexokinase and aldolase. D-Tagatose-6-phosphate was found to be phosphorylated by adenosine triphosphate at approximately the same rate as glucose-6-phosphate. The product, presumably a tagatose diphosphate, gives rise to triose phosphate.

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# A RAPID MICROMETHOD FOR ESTIMATION OF NON-VOLATILE ORGANIC MATTER\*

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A number of different methods for determining the purity of enzymes or other biologically active compounds have been used. Expression of purity as activity units per gm. of dry weight is not as useful for routine work as activity per gm. of organic matter because of the inorganic salts often used in purification procedures, and because inconveniently large samples are needed unless great care is used in weighing. The following have been used to determine the purity of enzymes: total nitrogen (1), protein by Kjeldahl analysis (2), by the biuret reaction (3), or by ultra-violet absorption (4). The method described below is rapid and convenient. A sample containing from 0.1 to 0.5 mg. of organic solids is required. The method has the additional advantage of responding to non-nitrogenous compounds. It has the disadvantage that it yields a somewhat arbitrary, although reproducible, figure. The method involves oxidation of the sample with dichromate in sulfuric acid, and determination of the excess dichromate colorimetrically or titrimetrically.

Determination of various organic compounds by measurement of dichromate consumed has often been used. A standard method (5) for cellulose and the method of Bloor (6) for lipides, are examples.

## *Reagents and Apparatus*

Only one reagent is needed. It is made by dissolving 5.00 gm. of  $\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$  in 20 ml. of water, and diluting to 1 liter with 95 per cent  $\text{H}_2\text{SO}_4$ . The reagent is stored in a glass-stoppered bottle protected from dust. A photoelectric colorimeter is desirable. If such an instrument is not available, 0.005 N thiosulfate, prepared less than 2 hours before use by dilution of standard 0.1 N thiosulfate, is needed for titration of the excess dichromate.

## *Procedure (Colorimetric Estimation)*

The aqueous sample, 0.4 ml. or less in volume, containing between 100 and 500  $\gamma$  of organic solids, is pipetted into a test-tube. Sufficient

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

water is added to bring the total volume to 0.4 ml. 1 ml. of oxidizing reagent is then added, and the contents of the tube well mixed. The tube is then heated in a boiling water bath for exactly 20 minutes, and cooled immediately. In addition to the samples, two blanks, with 0.4 ml. of water in place of the sample, are also heated. 10 ml. of water are added to each tube. To one of the blanks a small amount (roughly 10 mg.) of solid  $\text{Na}_2\text{SO}_3$  is added. A great excess over the amount necessary to reduce the dichromate should be avoided. After mixing, the solutions in the tubes are read in a photoelectric colorimeter equipped with a 440  $\text{m}\mu$  filter. The reduced blank is set at 100 per cent transmission (zero extinction) and the remaining tubes read against it.

### *Calculation*

The number of microequivalents (ml. of 0.001 N) of dichromate delivered by the 1 ml. pipette or burette used to add the oxidizing reagent to the sample is determined once for each lot of oxidizing reagent. (A convenient procedure is to deliver ten or twenty 1 ml. portions of oxidizing reagent into a flask, dilute, add a crystal of KI, and titrate with 0.1 N thiosulfate.) The blank tube, containing this amount of dichromate, is used as a colorimetric standard from which the amount of dichromate remaining in the sample tubes is calculated. Beer's law has been found to hold. For each microequivalent of dichromate consumed by the sample, 7  $\gamma$  of organic matter are assumed to be present in the sample (see Table I).

### *Titrimetric Procedure*

When a photometric instrument is not available, the samples and the blank, after heating, are rinsed into a flask with approximately 20 ml. of water, and, after addition of a small crystal of KI and thorough mixing, are titrated in the presence of starch indicator with 0.005 N thiosulfate. A reduced blank need not be prepared. The amount of organic matter in the sample is calculated from the titration difference between the blank and the sample on the assumption that 1  $\mu\text{eq.}$  of thiosulfate is equivalent to 7  $\gamma$  of organic solids.

### *Interfering Compounds*

Aqueous samples containing organic solvents cannot be used without previous evaporation of the solvent. With such samples, the tube containing the sample before addition of the oxidizing reagent, is placed in an oven until completely dry. Any other drying method that avoids charring is satisfactory. Water (0.4 ml.) is then added, and the determination carried out as usual. It has been found that the very thin layer of organic

matter left in the tube after drying dissolves readily in the oxidizing reagent, and the result is identical with that obtained on undried samples containing no organic solvent.

It is obvious that samples containing inorganic reducing agents (sulfite, cyanide, etc.) cannot be used. Organic reducing agents such as ascorbic acid or cysteine do not interfere, but are merely determined as a part of the organic matter of the sample.

### *Effect of Variations in Conditions*

*Composition of Sample*—Table I shows the number of micrograms of various biological materials corresponding to 1  $\mu$ eq. of dichromate. It will be noted that the figure is relatively constant for carbohydrate and

TABLE I  
*Reduction Equivalents of Biological Materials*

Material	Weight of sample*	Dichromate consumed	Micrograms per $\mu$ eq. dichromate
	$\gamma$	$\mu$ eq.	
Bakers' yeast . . . . .	524	68.5	7.65
Casein . . . . .	398	53.5	7.45
Blood serum (calf) . . . . .	451	67.4	6.70
Sucrose . . . . .	408	55.4	7.38
Corn-starch . . . . .	556	78.4	7.10
Soy bean phospholipide . . . . .	300	65.7	4.56
Butter fat (saponified) . . . . .	188.2	50.0	3.76
Corn oil " . . . . .	183.8	51.7	3.55

\* Dry, ash-free basis.

protein materials. Lipides, however, give lower figures. With the exception of phospholipides, however, such materials are not likely to be present in aqueous samples. The figure of 7.0  $\gamma$  per  $\mu$ eq. was arbitrarily adopted as being probably typical of the material most likely to be present in crude enzyme preparations. For some purposes, when the general type of organic matter present is known, it would be advantageous to use a factor experimentally determined for the type of material present.

*Amount of Sample*—In Fig. 1, Curve A shows the relation between the size of the sample and the amount of dichromate consumed for sucrose and casein samples. The relation is linear to at least 500  $\gamma$  of sample.

*Time of Heating*—Curves B of Fig. 1 show the effect of variations in heating time. It will be noted that after 20 minutes the rate of further oxidation is very small. In general, carbohydrate and lipid materials were found to be oxidized more rapidly than proteinaceous samples.

*Amount of Water Present*—The volume of liquid sample to be used

(0.4 ml.) was selected because, at the water concentration thus obtained, oxidation is more rapid than at lower or higher water concentrations. The data shown in Curve C, Fig. 1, are in accord with the results obtained by Snethlage (7) on a number of pure organic compounds.

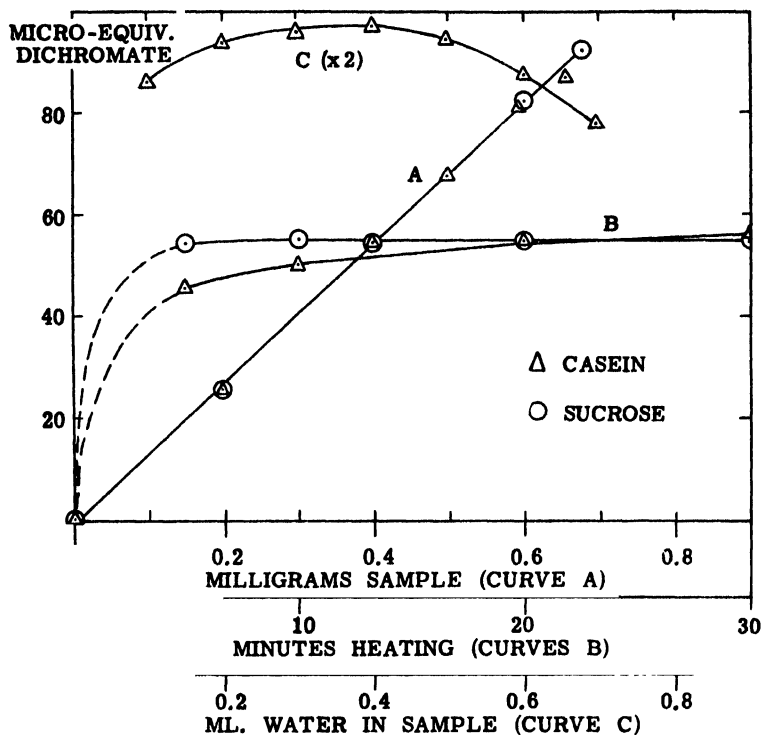


FIG. 1. Effect of variables on oxidation of casein and sucrose. The oxidation conditions were those of the standard procedure unless otherwise specified. Sample weights are on the dry, ash-free basis. The total dichromate present was 97  $\mu$ eq. Curve A, relation between weight of sample and amount of dichromate reduced. Curves B, effect of variation in heating time; sample, 408  $\gamma$  of sucrose or 398  $\gamma$  of casein. Curve C, effect of sample volume; sample, 381  $\gamma$  of casein. The heating time was reduced to 10 minutes to accentuate differences in oxidation rate. The amounts of dichromate reduced are plotted at twice their actual values.

### *Reproducibility*

In the colorimetric procedure, when all samples are read in the same colorimeter tube on the Evelyn colorimeter, the average deviation of replicates from the mean has been approximately 0.5 per cent of the original dichromate present. In the titrimetric procedure, the deviations have been of the same magnitude, but determinations have not been run

over a period long enough to establish an accurate estimate of the variability to be expected.

#### SUMMARY

A rapid method for approximate determination of non-volatile organic matter (100 to 500  $\gamma$ ) is described. The method involves colorimetric or titrimetric determination of dichromate remaining after the sample has partially reduced a sulfuric acid-dichromate oxidizing reagent.

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# A COLORIMETRIC METHOD FOR THE ESTIMATION OF URACIL AND CYTOSINE\*

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Until recently, the estimation of uracil and cytosine has depended on isolation procedures, with the exception of a manometric method for cytosine which is based upon the deamination of this pyrimidine by nitrous acid (1). Within the past 2 years, a microbiological method (2) and a spectrophotometric method (3-5) for the estimation of uracil, cytosine, and thymine have been reported. In addition, a colorimetric method for the determination of thymine is now available (6).<sup>1</sup>

The method to be described below is based upon the fact that uracil and cytosine, after bromination, reduce a uric acid reagent. Several workers in the past have studied the reactions of pyrimidines with the phenol and uric acid reagents (7-9). Our procedure may be considered to be an extension of the Wheeler-Johnson test for uracil and cytosine (10). In their test, the pyrimidine, in aqueous solution, is treated with bromine water, the excess bromine removed by aeration, and a solution of barium hydroxide is added. A purple precipitate is formed. We have found that, if after removal of the excess bromine, the uric acid reagent of Newton is added under the conditions described below, the reduction of the reagent becomes a quantitative measure of the pyrimidine present. Thus, 6 to 33  $\gamma$  of uracil or cytosine may be determined. Thiouracil may also be estimated by this method.

If we are dealing with a mixture of uracil and cytosine, the cytosine may be completely removed by adsorption on Amberlite IR-100-H. This resin, however, also picks up a small quantity of uracil. For the quantitative analysis of both pyrimidines, the synthetic zeolite, Decalso, is used as the adsorbent. It removes cytosine quantitatively, whereas uracil is not removed.

## EXPERIMENTAL

### *Reagents—*

Uric acid reagent. The stock solution of the lithium arsenotungstate reagent of Newton (11).

\* A preliminary report of this investigation was presented before the Division of Biological Chemistry of the American Chemical Society at Chicago, September, 1946.

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<sup>1</sup> Pircio, A., and Cerecedo, L. R., unpublished work.

**Urea-cyanide solution.** A 2.5 per cent sodium cyanide solution containing 25 per cent urea.

**Bromine water.** A saturated solution of bromine in water.

**Standard pyrimidine solutions.** Stock solutions (0.002 M) are prepared by dissolving pure cytosine monohydrate (25.8 mg.) and pure uracil (22.4 mg.) each in 100 ml. of distilled water. Dilute solutions for use are 0.0002 M or 0.0003 M.

**Amberlite IR-100-H.<sup>2</sup>**

**Decalso.<sup>3</sup>** This material is prepared for use as follows (12): The zeolite (60 to 80 mesh) is treated by stirring or shaking for 15 minutes with four portions (each of 8 to 10 volumes) of a 3 per cent acetic acid solution. Between the third and fourth washing a 20 minute treatment with 4 volumes of 25 per cent potassium chloride is carried out. The Decalso is then repeatedly washed with distilled water, filtered, air-dried, and bottled.

### *Procedure*

The unknown containing between 0.05 and 0.3  $\mu\text{M}$  of pyrimidine in neutral aqueous solution is measured into a test-tube calibrated to 25.0 ml. The volume is brought to 2 ml. with distilled water, and 7 drops of bromine water are added. After standing for exactly 5 minutes, the excess bromine is removed by aeration, and the aeration tube rinsed down with approximately 3 ml. of water. 5 ml. of the urea-cyanide solution are added, followed by 1.5 ml. of Newton's reagent. The tubes are shaken and allowed to stand for 1 hour or more for color development. After dilution to the 25 ml. mark, the solutions are read in the Evelyn photoelectric colorimeter with a 660 or 690  $m\mu$  filter. Standard solutions of the respective pyrimidine, as well as a reagent blank, are carried along with those of the unknown. All solutions should be kept at room temperature. Curves obeying the Beer-Lambert law are obtained when densities are plotted against the concentrations. The densities may vary with each batch of uric acid reagent.

### *Results*

In Tables I to IV are presented the results of experiments carried out with pure standard solutions of the respective substances tested under the conditions outlined for the quantitative estimation of cytosine and uracil. Table I shows the color development before and after bromination of some of the more important pyrimidines. None of these substances reduce the uric acid reagent before bromination. It should be noted that thymine

<sup>2</sup> Supplied by Eimer and Amend, New York.

<sup>3</sup> Supplied by The Permutit Company, New York.

and 5-methylcytosine are not chromogenic. All of the chromogenic compounds, uracil, cytosine, isocytosine, and thiouracil, obey the Beer-Lambert law. Thus, this method will serve to estimate these substances when they are present alone in solution.

Table II shows the color produced by various halogenated pyrimidines related to uracil and cytosine. Whereas the monobromo derivatives of uracil and cytosine do not reduce the reagent, the dibromo compounds are active in this respect. Similar findings were reported by Wheeler and

TABLE I

*Color Production by Various Pyrimidines of Biological Importance*

Concentration, 0.3  $\mu\text{M}$ ; filter, 660  $\text{m}\mu$ .

Substance	Density ( $2 - \log G$ ), after bromination
Uracil.....	0.99
Thymine.....	0
Cytosine.....	0.41
5-Methylcytosine.....	0
Isocytosine.....	0.39
Thiouracil.....	0.36

TABLE II

*Color Production by Certain Derivatives of Uracil and Cytosine*

Concentration, 0.3  $\mu\text{M}$ ; filter, 660  $\text{m}\mu$ .

Substance	Density ( $2 - \log G$ )	
	Before bromination	After bromination
5-Bromouracil.....	0	1.00
5,5-Dibromooxyhydrouracil.....	0.92	0.94
5,5-Dichlorooxyhydrouracil.....	0.14	0.14
5-Bromocytosine.....	0	0.27

Johnson in connection with their color test. They claimed that the colored product formed was the barium salt of dialuric acid, resulting from the alkaline hydrolysis of the dibromooxyhydrouracil (formed from both cytosine and uracil) to isodialuric acid and the rearrangement of the latter. Since, under the conditions of the quantitative method, neither isodialuric acid nor dialuric acid produces color in the same concentration range (either before or after bromination), as do cytosine and uracil, they cannot be the color-producing compounds formed from the cytosine and uracil. On the other hand, two facts indicate that the dibromooxyhydro derivatives of uracil and cytosine are directly responsible for the reduction of the

reagent. First, dibromooxyhydrouracil reduces the uric acid reagent to the same extent as does uracil when brominated under the conditions of the quantitative estimation. Second, Amberlite IR-100-H removes about 85 per cent of the brominated cytosine compound but less than 10 per cent of the brominated uracil derivative, which strongly suggests that the cytosine derivative still retains its amino group.

### *Interfering Substances*

The color production of some pyrimidines related to uracil and cytosine when subjected to the test is shown in Table III. Of these, only 5-nitrouracil and isobarbituric acid reduce the reagent in the 0.2  $\mu\text{M}$  range, and this activity is present only before bromination. Therefore, these compounds would not interfere after being carried through the regular

TABLE III

*Color Production of Some Pyrimidines Related to Uracil and Cytosine*  
Concentration, 0.3  $\mu\text{M}$ ; filter, 660  $\text{m}\mu$ .

Substance	Density ( $2 - \log G$ )	
	Before bromination	After bromination
5-Nitrouracil . . . . .	0.38	0
Isodialuric acid . . . . .	0	0
Dialuric acid . . . . .	0	0
Isobarbituric acid . . . . .	1.05	0.11
Barbituric acid . . . . .	0	0
Alloxan . . . . .	0	0

bromination procedure. Since the test is based on the reduction of the arsenotungstate reagent by a compound capable of being oxidized simultaneously, it is obvious that some of these substances are oxidized by the preliminary bromination to substances which are no longer able to reduce the reagent. Thus, 5-nitrouracil and isobarbituric acid lose their capacity to produce color after the treatment with bromine. This principle applies also to other compounds of biological interest, such as cysteine and reduced glutathione, which reduce the arsenotungstate reagent directly, but are oxidized on treatment with bromine. Similarly, uric acid loses its reducing capacity after bromination.

Of the purines to be found in nucleic acid hydrolysates, namely, adenine and guanine, the former produces no color either before or after bromination, whereas guanine, in 0.2  $\mu\text{M}$  quantities, although producing little or no color directly with the arsenotungstate reagent, does give a significant reduction after bromination at pH 7. It has been found in this labora-

tory (13)<sup>1</sup> that the test can be applied to nucleic acid hydrolysates after removal of the purines with palladous chloride (14).

### *Analysis of Mixtures of Uracil and Cytosine*

In our preliminary communication, we reported what was thought to be a clear cut separation of cytosine from uracil by means of the resin Amberlite IR-100-H, which can rapidly and quantitatively remove mg. quantities of cytosine. More recently, when an analytical grade of this resin, more finely divided than earlier samples, became available, the problem of the separation of the two pyrimidines was investigated more carefully. We found that under the conditions for the analysis of mixtures,

TABLE IV  
*Recovery of Cytosine and Uracil from Mixtures*

Composition of mixture (per 10 ml. of solution)		Per cent recovery	
Uracil	Cytosine	Uracil	Cytosine
$\mu M$	$\mu M$		
1.53		100	
2.04		102	
1.22	0.84	98	103
1.18	1.64	104	106
0.81	0.69	104	91
2.04	1.71	98	97
2.04	1.71	101	96
0.41	1.03	107	94
0.40	1.44	100	101
	1.80		98

as specified below, from 5 to 10 per cent of the uracil was removed in addition to the cytosine. Thus, while recoveries of uracil from mixtures are satisfactory, the values for cytosine become inaccurate, especially when we are dealing with mixtures in which the ratios of uracil to cytosine are large, because the optical density of uracil is about twice that of cytosine. This procedure may still be used, however, if one is interested only in ascertaining the amount of uracil present in a mixture.

The conditions for the analysis of mixtures are as follows: Mixtures of cytosine and uracil containing 1.5 to 4  $\mu M$  per 10 ml. of neutral aqueous solution are taken for analysis.

*Step 1*—A suitable aliquot (0.5 or 1 ml.) of the mixture is examined for its color production due to both uracil and cytosine according to the method described above.

*Step 2*—5 ml. of the mixture are placed in a 15 ml. centrifuge tube, 2

gm. of Decalso are added, and the tube is stoppered with a clean dry rubber stopper, and shaken for 5 minutes by hand. The zeolite is then removed by centrifugation.

*Step 3*—A suitable aliquot of the filtrate is taken for analysis. The color production is due to uracil alone.

A blank and standards for uracil and cytosine are run along with the analyses of the unknowns. From the dilution factor in Step 3, the optical density of the uracil in 1 ml. of the original mixture is calculated and this value is subtracted from that obtained in Step 1. The difference corresponds to the density of cytosine in 1 ml. of the original mixture.

The per cent recoveries for a series of mixtures analyzed in this manner are shown in Table IV.

The above method has been used in this laboratory for the determination of the pyrimidine constituents of yeast ribonucleic acid (13), and of the cytosine content of a desoxyribonucleic acid.<sup>1</sup>

#### SUMMARY

A colorimetric method for the determination of uracil and cytosine has been described. It is based upon the fact that these two pyrimidines, after bromination, reduce the uric acid reagent of Newton.

The quantitative removal of cytosine by Amberlite IR-100-H makes possible the determination of uracil in mixtures of these pyrimidines, and with the aid of the zeolite Decalso both can be estimated.

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## A PEPTIDE FROM A MARINE ALGA\*

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Haas and Hill (1) have reported that aqueous extracts of the brown marine alga *Pelvetia canaliculata* give a positive biuret reaction. These workers succeeded in isolating from such extracts a non-crystalline product which, on acid hydrolysis, yielded an appreciable quantity of glutamic acid of unstated configuration. They suggested, on the basis of a preliminary examination of the product, that it was an octapeptide but, in view of its impure state, refrained from further speculation as to its structure. In subsequent publications (2, 3), these investigators described the isolation of amorphous preparations of peptides from extracts of several other marine algae. A variety of amino acids were identified after acid hydrolysis, and the ratio of amino N before and after hydrolysis suggested that the peptides had chain lengths ranging from 2 to 11 amino acid residues.

The present investigation had as its initial objective the determination of the configuration of the glutamic acid identified by Haas and Hill in hydrolysates of the peptide-like material from *Pelvetia canaliculata*. Since this alga is not available in the United States, the closely related seaweed *Pelvetia fastigiata* was selected for study. We are greatly indebted to Dr. Paul C. Silva of the Department of Botany, University of California, for the collection of a quantity of this material at La Jolla, California, in the middle of the littoral zone, on December 27, 1948. After collection, the seaweed was dried and shipped to New Haven. Since aqueous extracts were found to give a strong biuret reaction, an effort was made to isolate a peptide by the procedure of Haas and Hill (1). In view of the brevity of the description given by these authors, the method of preparation employed in the present study is presented in greater detail in the experimental section.

It was a source of some satisfaction that the method of Haas and Hill, when applied to extracts of *Pelvetia fastigiata*, led to the isolation of a solid product, albeit of indefinite crystalline form. The product usually contained an appreciable quantity of inorganic matter (2 to 8 per cent), which

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could be largely removed by fractional precipitation with alcohol and ether, precipitation of sulfate ions with  $\text{BaCl}_2$ , and treatment with the cation exchange resin Amberlite IR-1.

The data presented in the experimental section appear to justify the conclusion that the isolation procedure had yielded a single substance of reproducible elementary composition and optical activity. The sodium fusion test for the presence of sulfur, phosphorus, or halogens proved to be negative, and it may be assumed, therefore, that the isolated material contains only carbon, hydrogen, oxygen, and nitrogen. The analytical data for the air-dried substance agree satisfactorily with the empirical formula  $\text{C}_{15}\text{H}_{25}\text{O}_8\text{N}_5$ . After being dried at  $100^\circ$  over  $\text{P}_2\text{O}_5$  *in vacuo*, the substance lost 1 molecule of water of crystallization per unit of the above elementary composition. The chemical studies to be described in what follows were conducted with samples of the substance that had been dried to constant weight in air.

The substance was found to be acidic in nature, and an aqueous solution had a pH of about 3. The determination of the neutral equivalent, by titration with 0.0147 N potassium hydroxide in 90 per cent ethanol (4), gave a value of  $408 \pm 4$  (average of four determinations). It would appear, therefore, that the substance contains one titratable acidic group per unit of elementary composition  $\text{C}_{15}\text{H}_{25}\text{O}_8\text{N}_5$ .

An aqueous solution of the substance failed to give a color with ninhydrin, and a quantitative determination of free  $\alpha$ -amino acids by the manometric ninhydrin method (5) showed the preparation to contain no more than about 0.04 per cent carboxyl N.

The presence of amide N was demonstrated by treatment with N sodium hydroxide in Conway vessels, according to the method of Warner and Cannan (6). In this manner, it was found that the substance contains 6.9 per cent amide N; the ratio of amide N to total N was 39.9 per cent. It may be concluded, therefore, that 2 of the 5 nitrogen atoms in the unit  $\text{C}_{15}\text{H}_{25}\text{O}_8\text{N}_5$  represent amide N.

On treatment with nitrous acid (7), the substance gave an amount of  $\text{N}_2$  corresponding to only 0.56 per cent amino N (3 minute reaction period; average of three determinations). On the other hand, after treatment with alkali as described in the preceding paragraph, the deamidated substance reacted more extensively with nitrous acid; the ratio of amino N to total N was found to be 34 per cent. These data led to the conclusion that, for every 3 nitrogen atoms which remained after the deamidation of the substance, one was a free amino group; the slight reaction of the intact material with nitrous acid was interpreted as due to the slow formation of free amino groups in the course of the determination. Evidence



for the correctness of this view will be presented in a later section of this communication.

A sample of the substance was subjected to hydrolysis with 8 N hydrochloric acid for 12 hours at 110°, and the hydrolysate was found to contain an amount of ammonia N which corresponded to 39.8 per cent of the total N. This value agrees satisfactorily with that obtained for the ratio of amide N to total N of the intact substance, and suggests that the ammonium ions in the hydrolysate arose from the cleavage of the amide groups. After removal of ammonia from the hydrolysate, the nitrogen which remained was found to be  $\alpha$ -amino acid N, since it could be accounted for completely as carboxyl N by the ninhydrin method or as amino N by the nitrous acid method.

Examination of the hydrolysate by means of paper chromatography with phenol-KCN (8) showed the presence of a single ninhydrin-reactive spot with a rate of migration identical with that of glutamic acid. Paper chromatography of another hydrolysate, which had been heated at 110° for only 3 hours, showed the presence of glutamic acid and of a slower moving component with an  $R_f$  value identical with that of an authentic sample of  $\alpha$ -L-glutamyl-L-glutamic acid (9). It was concluded, therefore, that the substance isolated from *Pelvetia fastigiata* was a peptide which, on complete acid hydrolysis, yielded glutamic acid as the major product. Accordingly, a quantitative estimation of the glutamic acid content of the 12 hour hydrolysate was performed by means of the glutamic acid decarboxylase of *Escherichia coli* (10), a generous sample of which was kindly placed at our disposal by Dr. I. C. Gunsalus and Dr. M. J. Blish, and it was found that 95.8 per cent of the nitrogen of the hydrolysate, after removal of ammonia, was represented by L-glutamic acid N. The conclusion as to the configuration of the glutamic acid is based on the available data on the optical specificity of the bacterial amino acid decarboxylases (11). In view of the likelihood that slight racemization had occurred during acid hydrolysis (12), it appeared justifiable to conclude that the peptide contains only glutamic acid residues, and that they are all of the L configuration. Analysis of the glutamic acid content of the hydrolysate by the method of Olcott (13) indicated that at least 81 per cent of the carboxyl N was represented by glutamic acid N. As shown by Kibrick (14), the completeness of the conversion of glutamic acid to pyrrolidonecarboxylic acid in this method depends greatly on the experimental conditions employed; this value can therefore only be considered as a minimal figure for the glutamic acid content of the hydrolysate. Conclusive evidence for the presence of L-glutamic acid in the acid hydrolysate was provided by the isolation of a sample of L-glutamic acid hydrochloride



liquid ammonia. These authors stated that the product was strongly levorotatory, but presented no analytical data as to its elementary composition. The procedure described in the present communication led to a racemic product, however, since it was optically inactive and, on hydrolysis, was converted to DL-glutamic acid.

It was of interest to find that IV, on treatment with  $N$  NaOH under the conditions employed for the deamidation of the peptide, was converted to a product which had an amine group that reacted quantitatively in the nitrous acid determination. It may be concluded, therefore, that the pyrrolidone ring of IV had been opened by alkali, and this finding may be offered in support of the view that the peptide from *Pelvetia fastigiata* also contains such a pyrrolidonoyl group. Furthermore, the intact pyrrolidonecarboxylic acid amide was found to react slightly with nitrous acid during the 3 minute period of treatment, and the amino N value indicated that the ring had been opened to the extent of about 11 per cent in the course of the determination. If structure I is accepted for the peptide, the amino N value of 0.56 per cent, mentioned earlier, would correspond to the opening of the pyrrolidone ring to the extent of about 16 per cent. This slight decomposition, in the presence of nitrous acid, of the pyrrolidone ring in compounds in which the 2-carboxyl group is present as an amide may be contrasted with the reported stability of pyrrolidonecarboxylic acid under similar conditions (16).

In offering formula I as the suggested structure of the peptide from *Pelvetia fastigiata*, it is recognized that the chemical data presented in this communication also permit the formulation of the peptide as one of the isomers of I which differ from I in the position of the amide groups and in the mode of linkage between the glutamic acid residues. Five such isomers may be mentioned:

- (a) L-Pyrrolidonoyl- $\alpha$ -L-glutaminylo-L-glutamine
- (b) L-Pyrrolidonoyl- $\alpha$ -L-glutamyl-L-glutamic acid diamide
- (c) L-Pyrrolidonoyl- $\gamma$ -iso-L-glutaminylo-L-glutamine
- (d) L-Pyrrolidonoyl- $\gamma$ -iso-L-glutaminylo-L-glutamine
- (e) L-Pyrrolidonoyl- $\gamma$ -L-glutamyl-L-glutamic acid diamide

A decision as to the mode of linkage between the two glutamic acid residues attached to the pyrrolidonoyl group would, of course, be made possible by the isolation of the tri-L-glutamic acid formed upon treatment of the peptide with  $N$  NaOH. If  $\alpha$ -L-glutamyl- $\alpha$ -L-glutamyl-L-glutamic acid were identified as the product of the alkaline treatment, the possibilities (c), (d), and (e) would be excluded, and the problem of the detailed structure of the intact peptide would revolve about the question as to the location of the amide groups. Regrettably, the amount of material available to us did not permit an attempt at the isolation of the alkaline degradation

product and its comparison with authentic samples of the isomeric tri-L-glutamic acid peptides.

It may be added that the fact that the product of alkaline treatment is a tripeptide, when considered in conjunction with the data on the neutral equivalent of the *Pelvetia* peptide, indicates that, under the conditions of the deamidation, the alkali did not cause extensive hydrolysis of the peptide linkages between the amino acids. This conclusion is supported by the finding that, in the solution of the deamidated peptide, the  $\alpha$ -amino acid N, as measured by means of the ninhydrin method, represented less than 5 per cent of the total N of the solution.

While the unequivocal proof of the structure of the product from *Pelvetia fastigiata* must await further chemical studies, and ultimate complete synthesis, it seems justifiable to view with greater favor the formulation of the peptide as I, rather than the other possibilities mentioned above. The wide-spread occurrence of L-glutamine in plant tissues (16) makes it reasonable to assume that a portion of this amino acid may be bound in peptide linkage in the case of *Pelvetia fastigiata*, and perhaps in other organisms. To our knowledge, no evidence is at hand for the occurrence of iso-L-glutamine, either free or combined, in biological systems; all the isomers of I, listed above, represent derivatives of this  $\alpha$ -amide.

It appears likely that the pyrrolidone ring in the peptide may have been formed in the course of the manipulations which led to its isolation, and that, in the living alga, there is present tri-L-glutamine which was converted to I by the elimination of ammonia. This view gains support from the experiments of Melville (17) and of Chibnall and Westall (18) who showed that, in synthetic glutamine peptides, when a glutamine residue provides the free  $\alpha$ -amino group of a peptide, the  $\gamma$ -amide group is extremely labile; substitution of the  $\alpha$ -amino group of glutamine, however, greatly stabilizes the amide linkage.

The authors wish to thank Miss Mary E. Mycek for her valuable assistance in this investigation.

#### EXPERIMENTAL

*Isolation of Peptide from Pelvetia fastigiata*—The dried seaweed (400 gm.) was ground and extracted with three successive 800 cc. portions of petroleum ether (30–60°). After being dried at room temperature, the defatted material was stirred with 1700 cc. of hot water for 1 hour, and the temperature was maintained at about 80° on the steam bath. The mixture was filtered through cheese-cloth, and reextracted twice more with 850 cc. portions of hot water. The combined filtrates (1900 cc.)

were poured into an equal volume of absolute ethanol. After 2 days, a gummy precipitate was removed by filtration with Filter-Cel. To the clear filtrate, 235 gm. of basic lead acetate were added with vigorous shaking. The mixture was chilled, filtered through Filter-Cel, and the filtrate was treated with an additional 75 gm. of basic lead acetate. After filtration, the filtrate was freed of lead by the addition of 67 cc. of 10 N sulfuric acid. The lead sulfate was removed by filtration, washed with water, and the combined filtrates and washings were concentrated *in vacuo* to about 300 cc. An equal volume of absolute ethanol was added to the solution.

The peptide was precipitated by the addition of 5 cc. of glacial acetic acid and of 75 gm. of mercuric acetate. The mixture was kept at 0° overnight, and the precipitate was collected by filtration, washed with absolute ethanol and with ether, and dried in air. It was then stirred with 100 cc. of 50 per cent ethanol saturated with mercuric acetate, and collected and washed as before. The dried material (2.5 gm.) was suspended in 100 cc. of water and  $H_2S$  was passed through the mixture. After removal of  $HgS$  by filtration, the filtrate was concentrated *in vacuo* to a syrup (0.65 gm.) which was dissolved in 3.5 cc. of water. Upon the addition of 13 cc. of absolute ethanol, 30.5 mg. of material (largely ammonium sulfate) separated, which was removed by filtration. To the filtrate, there were added 25 cc. of absolute ethanol, followed by 25 cc. of ether, and the mixture was kept in the ice chest for 2 days. The precipitate which appeared was collected by filtration and dried (230 mg.). The product had a nitrogen content of 18.65 per cent, and an ammonia determination showed that about 10 per cent of the total N was in the form of ammonium ions. A solution of the material also gave a positive test for sulfate ions.

197 mg. of the crude material were dissolved in 10 cc. of water, and the sulfate ions were removed by the addition of a slight excess of saturated barium hydroxide solution, corresponding to 60 mg. of  $Ba(OH)_2 \cdot 8H_2O$ . The  $BaSO_4$  was removed by centrifugation, washed with water, and the combined supernatant and washings (volume, 20 cc.) were passed through a column (0.6 cm.  $\times$  19 cm.) of Amberlite IR-1 at a rate of 0.5 to 1.0 cc. per minute. The column was washed through with 80 cc. of water, and the eluate was evaporated *in vacuo* (bath temperature, 35–40°) to yield a solid residue which was transferred to the filter with 5 cc. of absolute ethanol, and dried in air. This product gave a negative test for ammonium ions. Yield, 86 mg.; m.p., 190–195°, with browning at 170°;  $[\alpha]_D^{25} = -43.7^\circ$  (1.9 per cent in water). The optical activity is given on an ash-free basis; the ash content of this preparation was 0.94

per cent. After being dried at 100° over  $P_2O_5$  *in vacuo*, the material gave the following analytical values, which are corrected for the ash content of the preparation.

$C_{15}H_{25}O_7N_5$ .	Calculated.	C 46.75, H 6.02, N 18.18
385.4	Found.	" 46.52, " 6.13, " 17.81

A second preparation, dried in a similar manner, was found to contain 46.86 per cent carbon, 6.01 per cent hydrogen, and 18.07 per cent nitrogen (corrected for 0.87 per cent ash). The analytical data for this preparation, dried to constant weight in air, were as follows:

$C_{15}H_{25}O_8N_5$ .	Calculated.	C 44.66, H 6.25, N 17.36
403.4	Found.	" 44.55, " 6.08, " 17.33

This preparation melted at 190–195°, and its optical rotation was  $[\alpha]_D^{23} = -44.0^\circ$  (2 per cent in water). A third preparation of the peptide, which gave analytical data similar to those presented above, had a specific rotation of  $[\alpha]_D^{20} = -46.5^\circ$  (0.9 per cent in water).

*Deamidation of Peptide*—43.7 mg. of the peptide (corresponding to 7.55 mg. of nitrogen) were dissolved in 9 cc. of water in the outer well of a large Conway vessel (diameter of the outer well, 10 cm., diameter of inner well, 5 cm.). 10 cc. of 2 per cent boric acid reagent (19) were placed in the center well, and 8 cc. of 1.88 N NaOH were added to the outer well. The vessel was covered immediately, and kept at room temperature (about 25°) for 24 hours. The contents of the center well were titrated with 0.00993 N hydrochloric acid, of which 21.61 cc. were required. This amount is equivalent to 3.00 mg. of ammonia N. Separate trial experiments showed that no additional ammonia was liberated after 24 hours.

The solution in the outer well was transferred to a 25 cc. volumetric flask, neutralized with hydrochloric acid, and diluted to the mark with water. The solution contained 4.545 mg. of nitrogen. Amino nitrogen determinations by the nitrous acid method (3 minute shaking period) were performed on 5 cc. aliquots and showed that the solution contained 1.545 mg. of amino N.

*Acid Hydrolysis of Peptide*—48.2 mg. of the peptide (corresponding to 8.45 mg. of nitrogen) were dissolved in 3 cc. of 8 N hydrochloric acid, and the solution was heated under a reflux in an oil bath (110°) for 12 hours. The hydrolysate was transferred to the outer well of a large Conway vessel with the aid of 5 cc. of water. 10 cc. of 2 per cent boric acid reagent were placed in the center well, and 3 cc. of 40 per cent NaOH were added to the outer well. The vessel was covered immediately, rotated, and allowed to stand at room temperature for 5 hours. 23.22 cc. of 0.0103 N hydro-

chloric acid were required to neutralize the solution in the center well; this corresponds to 3.35 mg. of ammonia N.

The ammonia-free hydrolysate in the outer well was transferred to a 25 cc. volumetric flask, neutralized with hydrochloric acid, and diluted to the mark with water. The solution contained 4.85 mg. of nitrogen. Determinations of the amino N content (by the nitrous acid method) and of the  $\alpha$ -amino acid N content (by the ninhydrin method) on aliquots of the solution showed that it contained 4.85 mg. of amino N and 4.81 mg. of carboxyl N.

*Identification and Determination of L-Glutamic Acid in Acid Hydrolysate of Peptide*—For the determination of the L-glutamic acid content of an acid hydrolysate by the decarboxylase method, 15.5 mg. of the peptide (corresponding to 2.67 mg. of nitrogen) were hydrolyzed with 8 N hydrochloric acid as described in the previous section. The volume of the neutralized hydrolysate was adjusted to 5 cc., and 0.25 cc. aliquots were introduced into the side bulbs of three Warburg vessels which contained, in the main room, 0.5 cc. of enzyme suspension (5 mg. of L-glutamic acid decarboxylase) and 1.25 cc. of 0.075 M phthalate buffer (pH 5.0). Three other Warburg flasks were prepared in a similar manner, except that, instead of the peptide hydrolysate, 0.25 cc. aliquots of a solution of L-glutamic acid (0.337 mg. of nitrogen per cc.) were added to the side bulbs. After equilibration at 38.5°, the extent of CO<sub>2</sub> evolution was measured in the usual manner. The CO<sub>2</sub> output in the case of the peptide hydrolysate was  $116.8 \pm 2.0$   $\mu$ l., and with the L-glutamic acid,  $128.3 \pm 1.0$   $\mu$ l. The peptide hydrolysate contained, therefore, 1.53 mg. of glutamic acid N. This value represents 95.8 per cent of the amino acid N content (1.60 mg.) of the hydrolysate.

For the determination of the glutamic acid content of the hydrolysate by the Olcott method, an acid hydrolysate of 36.8 mg. of the peptide was adjusted to pH 4 and a volume of about 8 cc., and autoclaved at 20 pounds per sq. in. and 120–125° for 6 hours. The volume was then adjusted to 10 cc., and determinations by means of the Van Slyke ninhydrin method showed the solution to contain 0.71 mg. of carboxyl N. Before being autoclaved, the hydrolysate was found to contain 3.68 mg. of carboxyl N.

The isolation of L-glutamic acid hydrochloride from the acid hydrolysate of the peptide was effected in the usual manner. From 104 mg. of the peptide, there were obtained, after two recrystallizations, 69 mg. of the product.

C<sub>5</sub>H<sub>10</sub>O<sub>4</sub>NCl (183.6). Calculated, N 7.63; found, N 7.71  
[ $\alpha$ ]<sub>D</sub><sup>20</sup> = +26.2° (0.9% in water)

*Synthesis and Properties of Pyrrolidonecarboxylic Acid Amide*—4 gm. of carbobenzoxy-L-glutamic acid diamide (20) were suspended in 20 cc.

of methanol, and subjected to hydrogenolysis with palladium black in the usual manner. After removal of the catalyst by filtration, the clear solution was concentrated under reduced pressure to yield a syrup which was caused to crystallize by the addition of absolute alcohol. Yield, 1.6 gm.; m.p., 98–100°. The product was L-glutamic acid diamide.

$C_8H_{11}O_2N_2$  (145.2). Calculated, N 28.9; found, N 28.8

A solution of 265 mg. of L-glutamic acid diamide in 5 cc. of water was heated under a reflux for 6 hours, and then concentrated to dryness *in vacuo*. Water was added and the solution was again concentrated *in vacuo*; this process was repeated four times. The crystalline product which resulted was dried over  $P_2O_5$  *in vacuo* and recrystallized from hot ethanol. Yield, 160 mg.; m.p., 170° (browning), 200–205° (decomposition). A 1.8 per cent solution of the substance in water had no optical activity.

$C_8H_9O_2N_2$  (128.1). Calculated, N 21.9; found, N 21.7

1 cc. of an aqueous solution of pyrrolidonecarboxylic acid amide (17.24 mg.) was added to 1 cc. of 2 N NaOH, and the mixture was kept at room temperature (about 28°) for 24 hours. The solution was neutralized with hydrochloric acid and the volume was adjusted to 5 cc. A determination of the amino N content of the solution by means of the nitrous acid method (3 minute reaction period) showed it to contain 0.369 mg. of amino N per cc. Upon complete cleavage of the pyrrolidone ring, there would have been expected 0.378 mg. of amino N per cc. The treatment of the intact amide with nitrous acid gave an apparent amino N value of 1.2 per cent. This is about 11 per cent of the result to be expected (10.9 per cent) upon complete opening of the pyrrolidone ring.

#### SUMMARY

A peptide has been isolated from aqueous extracts of the marine alga *Pelvetia fastigiata* and, on the basis of degradation studies, has been formulated as L-pyrrolidonoyl- $\alpha$ -L-glutaminy-L-glutamine.

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## QUINOLINIC ACID METABOLISM

### IV. URINARY EXCRETION BY MAN AND OTHER MAMMALS AS AFFECTED BY THE INGESTION OF TRYPTOPHAN

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Various species of animals have exhibited differences in nicotinic acid metabolism as evidenced by the variation in the types and amounts of urinary end-products following the administration of nicotinic acid or its amide. Studies of these variations have been reported (1-3) in which the amounts of nicotinic acid, nicotinamide, *N*<sup>1</sup>-methylnicotinamide, nicotinuric acid, and 1-methyl-6-pyridone-3-carboxamide in the urine were determined.

Since it was observed that tryptophan could replace nicotinic acid in the diet of the rat (4), several investigators have examined the effect of tryptophan on the urinary excretion of end-products of nicotinic acid in various mammals. In the rat (5, 6), man (7, 8), the cotton-rat and horse (9), the dog (10), and the calf (11, 12) tryptophan has been shown to increase the excretion of nicotinic acid and one or more of its metabolites. These and other studies have amply proved that tryptophan is a metabolic precursor of nicotinic acid. Apparently the ability of different species to accomplish this synthesis from tryptophan varies widely, as is illustrated by their relative susceptibility to a nicotinic acid deficiency with various levels of dietary tryptophan and by the magnitude of the changes in urinary excretion of nicotinic acid or its derivatives in response to tryptophan administration.

The rat excretes a substantial part of a test dose of tryptophan as quinolinic acid (13), which has been only partially measured as nicotinic acid by a number of investigators who have used dilute mineral acids for hydrolysis of urine samples. This and other evidence, which strongly suggests that quinolinic acid is a metabolic intermediate between tryptophan and nicotinic acid (14, 15), made it desirable to study the urinary excretion of quinolinic acid by a number of species which appear to be less efficient at converting tryptophan to nicotinic acid. The effect of feeding or injecting tryptophan on the excretion of quinolinic acid, nicotinic acid,

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and *N*<sup>1</sup>-methylnicotinamide was determined for man, the guinea pig, calf, lamb, and pig.

#### EXPERIMENTAL AND RESULTS

*Methods*—Urine collections were made the day before tryptophan administration to determine the normal excretion for the diets consumed. Following this control period a rather large dose or doses of tryptophan were given orally or by injection during the early portion of a 24 hour period. One or two urine collections were made on the 1st and 2nd days after the tryptophan period to ascertain the rate at which the excretion of metabolites returned to normal values. Nicotinic acid and quinolinic acid were determined by the differential microbiological assay with *Lactobacillus arabinosus* (16, 14). *N*<sup>1</sup>-Methylnicotinamide was estimated by the method of Huff and Perlzweig (17).

*Human Studies*—Four adult, male subjects, without special dietary control, were used. Following a 24 hour urine collection, five 1 gm. doses of DL-tryptophan were given orally at 4 hour intervals and urine was collected until 8 hours after the last dose. For two subjects urine was collected during the 24 hour period immediately following the day the tryptophan was given.

The results are shown in Table I. The excretion of nicotinic acid and its amide<sup>1</sup> was approximately 1 mg. per day for all subjects and was not affected significantly by the tryptophan ingestion. Quinolinic acid excretion was 3.1 to 5.5 mg. per day and rose to 10.6 to 11.9 mg. when tryptophan was ingested. The excretion the following day was reduced to values approaching the preexperimental control period in the two subjects studied. The *N*<sup>1</sup>-methylnicotinamide values were more variable, but in all cases the tryptophan caused increases. While the increased quinolinic acid excretion was relatively small compared to that of the rat, a highly significant increase ( $P < 0.001$ ) did occur. The increase represented only 0.18 per cent conversion of the tryptophan.

*Guinea Pigs*—Four guinea pigs approximately 3 months of age were used. They had received a purified ration containing 30 per cent casein for 8 weeks before the experiment. They were then fed a 9 per cent casein-sucrose ration low in nicotinic acid for 3 days prior to the control period. Three animals received 1 mm (204 mg.) of L-tryptophan in 0.9 per cent sodium chloride at pH 7 by intraperitoneal injection, in four equally spaced doses in 24 hours. The remaining animal received an equimolar amount of the sodium salt of quinolinic acid by the same route. Urine was collected at the end of this period, but that for the following 24

<sup>1</sup> Nicotinic acid values as used throughout include nicotinamide, nicotinuric acid, and any other compounds active for *Lactobacillus arabinosus*.

hour period was discarded. The 2nd day after the injections urine was again collected and analyzed.

TABLE I  
*Effect of DL-Tryptophan on Urinary Excretion of Its Metabolites by Human Male Subjects*

Subject No.	Age	Weight	Period of collection*	Excretion, mg. per 24 hrs.		
				Nicotinic acid	Quinolinic acid	N <sup>1</sup> -Methylnicotinamide
1	32 yrs.	180 lbs.	1	0.99	3.5	4.6
			2	1.1	10.6	6.3
			3	1.0	4.2	4.8
2	31	180	1	0.96	3.1	8.7
			2	0.96	11.9	9.8
3	31	160	1	0.87	3.1	8.2
			2	0.83	10.9	11.2
4	28	145	1	0.95	5.5	6.8
			2	0.97	11.5	10.2
			3	0.96	6.0	10.0

\* Period 1, before tryptophan administration; Period 2, during administration; Period 3, after administration.

TABLE II  
*Effect of Tryptophan and Quinolinic Acid on Urinary Excretion of Quinolinic Acid, Nicotinic Acid, and N<sup>1</sup>-Methylnicotinamide by Guinea Pig*

Guinea pig No.	Weight	Sex	Substance injected during 24 hr. urine collection	Excretion, mg. per 24 hrs.		
				Nicotinic acid	Quinolinic acid	N <sup>1</sup> -Methylnicotinamide
1	250 gm.	F.	None	0.029	0.36	0.050
			1 mm quinolinic acid	0.056	79.0	0.20
			None, 2nd day after injection	0.022	1.32	0.036
2	372	M.	"	0.072	0.125	0.030
			1 mm L-tryptophan	0.068	0.63	0.23
			None, 2nd day after injection	0.024	0.21	0.036
3	237	F.	"	0.050	0.135	0.035
			1 mm L-tryptophan	0.054	0.57	0.22
			None, 2nd day after injection	0.030	0.25	0.030
4	204	"	"	0.034	0.15	0.040
			1 mm L-tryptophan	0.071	0.45	0.17
			None, 2nd day after injection	0.026	0.21	0.040

The results are shown in Table II. Quinolinic acid excretion during the control period varied from 0.125 to 0.36 mg. per day, nicotinic acid

TABLE III

*Effect of Tryptophan on Urinary Excretion by Male Calf of Nicotinic Acid, Quinolinic Acid, and N<sup>1</sup>-Methylnicotinamide*

Calf No.	Breed	Age	Weight	24 hr. collection period*	DL-Tryptophan fed	Excretion, mg. per 24 hrs.		
						Nicotinic acid	Quinolinic acid	N <sup>1</sup> -Methylnicotinamide
		<i>days</i>	<i>lbs.</i>		<i>gm.</i>			
13	Holstein	20	128	1		1.47	8.42	2.21
				2	5	4.70	15.3	3.51
				3		2.88	7.63	1.61
				4		3.74	10.7	2.79
1	Brown Swiss	24	125	1		1.44	1.96	0.95
				2	5	4.39	8.74	2.04
				3		1.22	8.15	2.33
				4		1.74	6.90	2.10
11	Holstein	17	90	1		5.41	10.2	1.32
				2	5	11.3	61.7	2.08
				3		18.5	11.7	2.36
				4		12.3	4.17	3.82
20	Brown Swiss	18	117	1		5.41	8.78	1.79
				2	5	4.49	89.0	2.14
				3		9.74	18.6	2.32
				4		5.10	17.6	1.76

\* Period 1, before; Period 2, during; Periods 3 and 4, after tryptophan administration.

TABLE IV

*Effect of Tryptophan on Urinary Excretion by Female Lamb of Nicotinic Acid, Quinolinic Acid, and N<sup>1</sup>-Methylnicotinamide*

Lamb No.	Age	Weight	24 hr. collection period*	DL-Tryptophan fed	Excretion, mg. per 24 hrs.		
					Nicotinic acid	Quinolinic acid	N <sup>1</sup> -Methylnicotinamide
	<i>days</i>	<i>lbs.</i>		<i>gm.</i>			
1	100	25	1		1.58	3.47	0.85
			2	2	2.36	22.9	1.46
			3		0.20	6.90	0.85
			4		0.77	3.48	
2	106	38	1		4.65	2.42	1.21
			2	2	6.12	20.2	5.40
			3		7.52	9.15	1.12
			4		4.10	2.75	

\* Period 1, before; Period 2, during; Periods 3 and 4 after tryptophan administration.

from 0.029 to 0.072 mg., and N<sup>1</sup>-methylnicotinamide from 0.030 to 0.050 mg. Quinolinic acid administration increased the excretion of nicotinic

acid and *N*<sup>1</sup>-methylnicotinamide considerably, but approximately half of it was excreted promptly as such. All values returned toward those of the control period the 2nd day. The tryptophan had little, if any, effect on the nicotinic acid excretion, but increased the *N*<sup>1</sup>-methylnicotinamide

TABLE V

*Effect of Tryptophan on Urinary Excretion by Male Duroc Jersey Pig of Nicotinic Acid, Quinolinic Acid, and N<sup>1</sup>-Methylnicotinamide*

Pig. No. (age, 11 mos.)	Weight	24 hr. collection period*	DL- Tryptophan fed	Excretion, mg. per 24 hrs.		
				Nicotinic acid	Quinolinic acid	<i>N</i> <sup>1</sup> -Methyl- nicotinamide
	lbs.		gm.			
1	178	1		0.65	4.37	2.0
		2	5	4.55	60.0	5.3
		3		0.74	13.9	4.5
		4		0.67	12.3	1.1
2	178.5	1		0.78	6.68	2.5
		2	5	4.75	49.2	9.2
		3		0.9	14.0	7.4
		4		1.14	14.0	4.0
3	167	1		0.91	9.6	2.4
		2	5	7.30	50.2	3.6
		3		1.58	20.9	3.8
		4		0.72	14.8	3.5
4	179	1		0.78	9.4	2.7
		2	5	3.83	38.3	6.6
		3		0.79	13.8	4.3
		4		1.24	12.0	4.1
5	180.5	1		0.79	8.2	2.5
		2	5	14.25	15.1	6.9
		3		1.81	15.0	5.5
		4		0.85	14.7	3.1
6	169.5	1		0.69	2.64	3.6
		2	5	6.00	44.2	12.2
		3		0.99	11.7	5.2
		4		0.98	12.3	5.5

\* Period 1, before; Period 2, during; Periods 3 and 4, after tryptophan administration.

4- to 8-fold. Quinolinic acid excretion also increased 3- to 5-fold, then decreased toward control values the 2nd day following the tryptophan.

*Calves*—Four calves being raised on a synthetic milk diet (18) were studied. Following a 24 hour control period each calf received orally 5 gm. of DL-tryptophan at the beginning of the collection period. Urine was collected 24, 48, and 72 hours later. The results of the analyses are presented in Table III. The nicotinic acid increased from 3.43 mg.,

average, to 6.23 mg. during the administration of tryptophan, and continued to increase the day following in two of the four calves. Quinolinic acid excretion, which varied from 1.96 to 10.2 mg. per day, with an average of 7.33 mg., increased from 2- to 10-fold in response to tryptophan and remained slightly elevated the 2 following days. The amount of the compound measured as *N*<sup>1</sup>-methylnicotinamide by the method employed was not affected greatly by tryptophan. This is not unexpected, since Johnson *et al.* (19) have shown that the ingestion of nicotinic acid or its amide does not greatly influence the excretion of this compound by the calf. The increased excretion of nicotinic acid-like compounds and the corresponding increase in quinolinic acid in the urine are in good agreement with the results obtained with other species studied.

*Lambs*—Another polygastric animal was studied with essentially the same results (Table IV). Two lambs receiving a synthetic milk ration (18) were fed 2 gm. of DL-tryptophan mixed in the first of three feedings. The urinary excretion of these metabolites was determined as with calves. The tryptophan caused moderate increases in the nicotinic acid and *N*<sup>1</sup>-methylnicotinamide excretion and a 6- to 8-fold increase in quinolinic acid, which rapidly returned to approximately 3 mg. per day, the normal value.

*Swine*—Six 11 month-old, male pigs, consuming a good practical ration, were studied in the same manner as the other species, with similar results (Table V). 5 gm. of DL-tryptophan were given orally at the beginning of the 24 hour experimental period. The response to tryptophan was considerable in the case of all metabolites. Quinolinic acid excretion was slower in returning to normal values than were nicotinic acid and *N*<sup>1</sup>-methylnicotinamide. The control values for the latter are somewhat lower than those reported for younger pigs by Luecke *et al.* (20) but are in agreement with the values of 1.7 to 3.2 mg. per day for pigs receiving a normal diet supplying approximately 17 mg. of nicotinic acid per day (21).

#### DISCUSSION

These results indicate that quinolinic acid or some closely related substance is a normal component of the urine of a number of species. The increased amount excreted when tryptophan was ingested suggests that the dietary tryptophan is the precursor of the small amount of quinolinic acid normally present in the urine.

It is interesting to compare these five species with the rat in this respect. In Table VI the normal excretion values expressed as mg. of quinolinic acid per kilo of body weight, and the per cent of the additional tryptophan administered which was excreted as quinolinic acid, are given. Strict comparisons are not justified since the diets were not comparable and the



results would undoubtedly be affected by the level of dietary tryptophan, quinolinic acid, and nicotinic acid. The so called normal excretion values are possibly a function of the nitrogen metabolism. The guinea pigs were losing weight rapidly as a result of the low protein purified diet; hence the values could not be considered normal. Possibly the tryptophan from tissue breakdown is available for quinolinic acid formation. The responses to added tryptophan fell into three classes: man and the guinea pig near 0.2 per cent conversion, the calf, lamb, and pig near 1 per cent, and the rat 10 to 20 per cent. The rate of this transformation may play a rôle in determining the necessity of a dietary source of nicotinic acid for the monogastric animals.

TABLE VI  
*Comparative Excretion of Quinolinic Acid by Number of Species As Affected by Tryptophan*

Species	Control values per kilo body weight	During tryptophan administration	
		Dosage of tryptophan per 24 hrs.	Increased excretion, per cent of administered tryptophan
	mg.	gm. per kg.	
Man	0.05	0.066 Orally	0.18
Guinea pig	0.72	0.75 Intraperitoneally	0.25
Rat, 300 gm. (Henderson (14))	0.14	0.68 "	12.8
" 40-70 gm. (Henderson (14)).	0.058	0.060 "	18.8
Calf	0.14	0.096 Orally	0.90
Lamb	0.20	0.14 "	1.14
Pig	0.084	0.063 "	0.85

#### SUMMARY

1. A quantitative study has been made of the effect of tryptophan on the excretion of nicotinic acid, quinolinic acid, and *N*<sup>1</sup>-methylnicotinamide by man, the guinea pig, calf, sheep, and pig.

2. In general, the largest change was observed in the quinolinic acid excretion. It rose markedly during the tryptophan administration, then rapidly returned toward preexperimental values.

3. The per cent of the tryptophan which appeared as urinary quinolinic acid varied from approximately 0.2 per cent for man and the guinea pig to 10 to 20 per cent for the rat. The farm animals studied converted approximately 1 per cent of the additional DL-tryptophan fed.

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# FACTORS AFFECTING THE MAINTENANCE OF OXIDATIVE PHOSPHORYLATION IN A KIDNEY HOMOGENATE SYSTEM\*

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In earlier publications from this laboratory a system was described by which the ability of tissue homogenates to oxidize oxalacetate (1, 2) was measured. In these studies it was observed that the rate of oxygen uptake declined rapidly during the course of the reaction and failed completely in 2 hours or less. While measurement of the initial rate is adequate for assay purposes, it is of interest to investigate the factors involved in the maintenance of oxidation and the production of high energy phosphate. Potter found a greater lability of the enzymes required for oxidative phosphorylation (3) compared to those needed for non-phosphorylative oxidation of succinate (4) or malate (5). Studies have been made on the lability of enzymes which oxidize oxalacetate, both in tissues *in situ* and in homogenates at 0°, and the loss of oxidative ability was correlated with the loss of high energy phosphate (1). The present communication deals with factors involved in the maintenance of oxidative phosphorylation for 4 to 6 hours in homogenate systems.

## Methods

Male rats of the Holtzman strain, weighing 200 to 300 gm., were used. They were killed by decapitation, bled, and the required tissues were removed rapidly and placed in 8.5 per cent sucrose at 0° on cracked ice. Earlier work (1) made it appear that kidney was the most labile tissue; therefore it was used in the bulk of the present studies. Each sample was weighed and homogenized with 9 volumes of cold 8.5 per cent sucrose (6). The homogenate was pipetted into cold Warburg flasks containing the other components of the system and the flasks were shaken in the Warburg apparatus at 38°. Readings were taken at intervals and the average rate of oxygen uptake was plotted against time at the midpoint of each period. The maximum rate and the duration, expressed as

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the time at which the rate fell to half the maximum, were measured from the plot.

When analyses were desired, the flasks were set in cracked ice at the appropriate time and 2.0 ml. of cold 17.5 per cent trichloroacetic acid were added to each flask. The denatured protein was removed by centrifugation and the supernatant fluid was analyzed. The following analyses were made: true inorganic phosphate<sup>1</sup> (7, 8), citrate (9), keto acids (10), malate,<sup>2</sup> and ammonia (11). The pH was estimated visually with phenol red and was probably accurate to 0.1 to 0.2 unit.

The chemicals were commercial products of reagent grade, except for cytochrome *c*, ATP (40 per cent adenosine triphosphate, 60 per cent adenosine diphosphate), diphosphopyridine nucleotide (DPN), and triphosphopyridine nucleotide (TPN),<sup>3</sup> oxalacetate, and  $\alpha$ -ketoglutarate, which were prepared in this laboratory.

### Results

#### *Additions to Reaction Mixture*

**Standard Conditions**—Rather than present the numerous experiments which led to the conditions chosen for maximum duration and maximum rate, we give the final conditions below. These are followed by data concerning their variations separately, with other additions at the optimum levels. Other data were obtained under nearly optimum conditions and gave similar results which are not shown. The standard medium used throughout this investigation, unless otherwise stated, was as follows (final concentrations): gas phase, air containing 1 per cent CO<sub>2</sub> (12), 0.0027 M KHCO<sub>3</sub>, 0.017 M potassium phosphate (pH 7.0), 0.015 M potassium citrate, 0.0033 M potassium fumarate, 0.01 M potassium pyruvate, 0.0016 M K ATP, 0.2 ml. of rat kidney homogenate in 8.5 per cent sucrose, 0.06 M sucrose (not including sucrose in the homogenate), 0.0033 M MgCl<sub>2</sub>,  $2.7 \times 10^{-5}$  M cytochrome *c*, final volume 3.0 ml. All compounds except ATP were put into a stock solution to simplify pipetting. From observation of numerous duplicate determinations it was decided that a difference in rate of 5  $\mu$ l. of O<sub>2</sub> per 10 minutes or a difference in

<sup>1</sup> Lowry and Lopez (7) used 1 per cent ammonium molybdate in their work, but Potter (8) used 0.5 per cent to decrease the color produced by organic phosphates. Although 0.5 per cent ammonium molybdate was satisfactory for small amounts of phosphate, it was found to give a color not proportional to the amount of phosphate at higher concentrations; therefore 1 per cent ammonium molybdate was used in the present work.

<sup>2</sup> Speck, J. F., unpublished.

<sup>3</sup> We wish to thank Dr. G. A. LePage and Dr. G. C. Mueller for providing the samples of DPN and TPN.

duration of 20 minutes was significant in the comparison of two experiments when the same homogenate was used in both cases. In selecting the optimum conditions, both the maximum rate and the duration were considered.

*Buffer and CO<sub>2</sub>*—The optimum pH appeared to be 7.0 to 7.2; lower pH (6.8) decreased the initial rate from 48 to 39  $\mu$ l. per 10 minutes and higher pH (7.4) decreased the duration from 250 to 160 minutes. When NaOH was placed in the center well and the initial pH was 6.9 to 7.0 in the reaction mixture, the final pH was 7.4 to 7.5 in the presence of 0.017 M phosphate buffer. Increasing potassium phosphate to 0.033 M was not satisfactory because it decreased the initial rate from 42 to 36  $\mu$ l. per 10 minutes. When a diethanolamine-HCl-KHCO<sub>3</sub> solution was placed in the center well in place of NaOH so that a constant CO<sub>2</sub> pressure was maintained in the flask (12), the pH was more constant, and did not rise above pH 7.2 to 7.3 in the presence of 1 per cent CO<sub>2</sub>. However, a significant improvement in rate or duration was not observed.

The buffering capacity of the mixture in the center well is proportional to the CO<sub>2</sub> pressure and to  $1/H^+$  in the flask contents. The change of pH, in the absence of other buffers, per micromole of KHCO<sub>3</sub> produced per ml. is theoretically equal to  $2.7 \times 10^6 (H^+)/CO_2$ , where  $(H^+)$  is in moles per liter and  $(CO_2)$  is in per cent. The buffering capacity is equal to 0.02 M phosphate at pH 7.3 and 1 per cent CO<sub>2</sub>, and is better at higher pH. It should not be necessary to add other buffers above pH 7.5 in most experiments if a mixture is used in the center well that maintains 1 per cent CO<sub>2</sub>. The pH of the reaction mixture depends on the phosphates and KHCO<sub>3</sub> added.

*Substrate Levels*—The concentration of oxalacetate in the system devised to assay for oxalacetic oxidase (2) gave the optimum initial rate, but the amount of substrate was so small that it was soon used up. In order to prevent the reaction from stopping, various combinations of substrates were tried (Table I), and the combination selected is given under "Standard conditions." Early experiments showed that citrate was of primary importance in increasing duration and this is probably due to its effect on adenylic acid breakdown (see below). A very long lag was observed when fumarate was omitted; almost 3 hours were required before the rate reached that of the control, although the duration was little affected. Except for a lag in the absence of pyruvate, little effect was noted on its omission, and the level chosen was probably higher than necessary, but was not harmful. Addition of succinate gave a large initial rate but did not help duration, and the addition of 0.0033 M oxalacetate and 0.0067 M  $\alpha$ -ketoglutarate had a slightly harmful effect on the maximum rate (Table I).

Considerable amounts of substrate remained at the end of the reaction (Fig. 1), and therefore it can be concluded that oxygen uptake did not stop because of lack of substrate. It might be thought that the extended oxygen uptake was due to oxidation of citrate to  $\alpha$ -ketoglutarate, which was observed to build up gradually during the reaction, but this was

TABLE I  
*Effect of Variation of Substrates*

All conditions were standard except the concentration of the compound shown in Column 1. Column 2 shows the maximum rate of oxygen uptake in  $\mu$ l. of  $O_2$  per 10 minutes, and Column 3 shows the minutes required until the rate of oxygen uptake fell to one-half the maximum rate of the flask that was run under completely standard conditions (shown in bold-faced type). The numbers are averages of duplicate experiments.

Variation (1)	Maximum rate (2)	Duration (3)
	$\mu$ l $O_2$	min.
0.010 M citrate	46	210
0.015 " "	<b>44</b>	<b>270</b>
0.020 " "	40	280
0.025 " "	36	290
0 M fumarate	36	290
0.0033 M fumarate	<b>44</b>	<b>270</b>
0.0067 " "	42	250
0 M pyruvate	39	220
0.010 M pyruvate	<b>43</b>	<b>240</b>
0.0067 " "	46	270
0.010 " "	<b>44</b>	<b>270</b>
0.0133 " "	42	270
0 M succinate	<b>40</b>	<b>310</b>
0.017 M succinate	59	300
0 M oxalacetate + 0 M $\alpha$ -ketoglutarate	<b>40</b>	<b>310</b>
0.0033 M oxalacetate + 0.0067 M $\alpha$ -keto-glutarate	35	320

not supported by the data, for only 45 of the observed 260  $\mu$ l. taken up between 240 and 440 minutes can be accounted for in this way.

It was calculated that about 50 per cent of the oxygen in the flasks was used up in these experiments; so that if much longer runs were achieved oxygen would have to be replenished.

*High Energy Phosphate*—Table II shows that 0.0016 M ATP gave maximum rate and duration and that even a 5-fold reduction did not seriously harm the system. When adenylic acid was substituted for ATP, the system ran equally well or better initially, but not as long. This was also the case when adenylic acid was present in addition to ATP. Fluoride,

which inhibits ATP breakdown (8), was harmful. When  $3 \times 10^{-4}$  M 2,4-dinitrophenol, which inhibits phosphorylation (13), was added, no

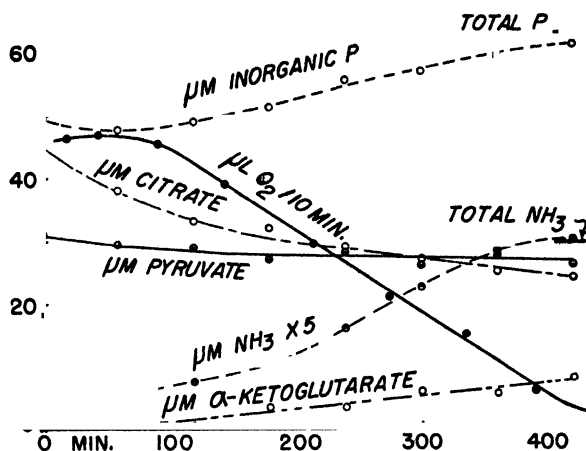


FIG. 1. Changes during reaction. The standard conditions were used. Initially  $10 \mu\text{M}$  of fumarate were present, and, in a separate experiment,  $12 \mu\text{M}$  of malate were found at the end of the reaction. The points are averages of duplicate experiments. The concentrations are in micromoles per flask. The figures on the ordinate scale represent micromoles of oxygen uptake in 10 minutes.

TABLE II

*Effect of Variation of ATP and Adenylic Acid*

The conditions and the column headings are the same as for Table I.

Variation (1)	Maximum rate (2)	Duration (3)
	$\mu\text{L. O}_2$	min.
0 M ATP	4	0
$3 \times 10^{-4}$ M ATP	38	215
$1.0 \times 10^{-3}$ M ATP	44	240
$1.6 \times 10^{-3}$ " "	48	250
$2.7 \times 10^{-3}$ " "	48	260
0 M adenylic acid	44	260
$4 \times 10^{-3}$ M adenylic acid	51	205
$2.7 \times 10^{-3}$ M adenylic acid + 0 M ATP	48	235
$6 \times 10^{-3}$ M KF	37	180
0 M 2,4-dinitrophenol*	43	190
$3 \times 10^{-4}$ M 2,4-dinitrophenol*	40	95

\*  $1.3 \times 10^{-5}$  M cytochrome c, 0.01 M fumarate, and 0.01 M citrate were used.

initial effect was observed but the system failed in 120 minutes, and at this time the ATP was gone, showing that if conditions are such that ATP is lost completely the oxidative ability is also lost.

Loss of ATP can cause the failure of oxygen uptake, or ATP can be lost as a result of failure of oxygen uptake due to failure of some other part of the reaction system. It can be seen from Fig. 1 that oxygen uptake decreased a considerable time before the high energy phosphate had fallen to a limiting level, as shown by the rises in inorganic phosphate. Thus the oxygen uptake did not decrease because the ATP was lost in the experiment described.

*Cytochrome c*—Little effect on the initial rate of the reaction was observed when cytochrome *c* was varied (Fig. 2). However, a great effect on duration was observed when the cytochrome *c* level was raised from zero to  $2.6 \times 10^{-5}$  M. The time required for oxygen uptake to fall to

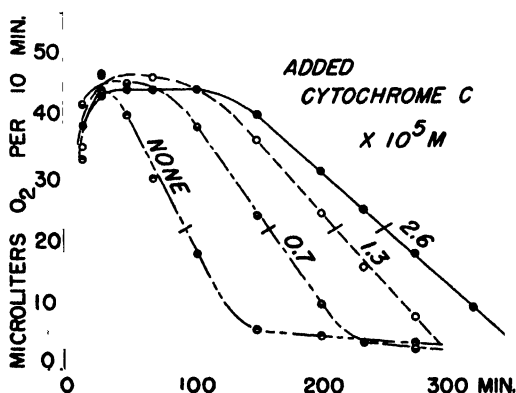


FIG. 2. Effect of cytochrome *c* on rate and duration of oxygen uptake. The standard conditions were used except for the concentration of cytochrome *c*. The points are averages of duplicate determinations. The intercepts of the curves with the short lines at 22  $\mu$ l. per 10 minutes represent the durations of the experiments.

half the maximum rate changed from 95 to 255 minutes. Increasing the concentration above  $2.6 \times 10^{-5}$  M did not further improve duration. The shorter duration in the absence of cytochrome *c* may be due to gradual loss of cytochrome *c* from the mitochondria. Addition of serum albumin had no effect, indicating that cytochrome *c* did not act merely as a non-specific protein.

*Tonicity*—Variation of the tonicity of the homogenate between 6 and 12 per cent sucrose or substitution of isotonic KCl for sucrose had little effect on the rate or duration. Addition of extra KCl to the reaction system was slightly inhibitory, probably because excess  $K^+$  was added as the cation for substrates and buffer. Addition of 0.06 M sucrose was helpful, however.

*Cofactors*—The  $MgCl_2$  level was optimum at 0.0033 M (Table III), as



it was in the system devised for assay of oxalacetic oxidase (2), though a large part (about 80 per cent) of the Mg must be in a complex with citrate (14) and this may reduce the rates of oxidation by enzymes requiring  $Mg^{++}$  (15). The various coenzymes had little effect on the initial rate of reaction. Both DPN and TPN greatly decreased duration. The reason for this is not clear, but it is very unlikely that metal contaminants were responsible and inhibition of bound coenzymes may be indicated.

TABLE III

*Effect of Addition of Cofactors*

The conditions and the column headings are the same as for Table I.

Variation (1)	Maximum rate (2)	Duration (3)
	$\mu l. O_2$	<i>min.</i>
0.0023 M $MgCl_2$	45	260
0.0033 " "	48	250
0.0033 " " *	45	210
0.0067 " " *	48	180
0 M nicotinamide (NA)	48	240
0.04 M nicotinamide	43	220
0 $\gamma$ DPN, 0 $\gamma$ TPN, 0.0 M NA*	38	190
400 $\gamma$ DPN, 0 $\gamma$ TPN, 0.04 M NA*	38	110
0 $\gamma$ DPN, 100 $\gamma$ TPN, 0.04 M NA*	42	110
0 $\gamma$ DPN, 0 $\gamma$ TPN, 0.0 M NA*	42	215
600 $\gamma$ DPN, 80 $\gamma$ TPN, 0.04 M NA*	35	80
No cofactors†	46	260
Cofactors‡	50	150

\*  $1.3 \times 10^{-5}$  M cytochrome c was used.

† 2 N NaOH in the center wells.

‡ 360  $\gamma$  of DPN, 30  $\gamma$  of TPN, 5 mg. of NA, 50  $\gamma$  of biotin, 100  $\gamma$  of pyridoxine, 100  $\gamma$  of cocarboxylase, approximately 1 unit of coenzyme A (obtained from Dr. F. M. Strong).

*Later Additions*—If the rate of oxygen uptake declines because of the loss of some compound originally in excess, later addition of the compound might be expected to restore the rate. Table IV gives data of this nature, showing that the rate of oxygen uptake was not helped by citrate, cytochrome c, or ATP, but was benefited, though not completely restored, by DPN. Addition of other cofactors to the DPN did not help further. Since DPN was not initially helpful, deterioration consists, at least partly, in breakdown of DPN originally in the homogenate. The stimulation by DPN was brief. In 1 hour the rate returned to that of the control.

*Citrate and Phosphorylation*—Citrate seems especially important for

TABLE IV

*Effect of Later Additions to Reaction System*

The standard conditions were used, except that the volume was 2.8 to 3.0 ml. before the addition and 3.0 to 3.3 ml. afterwards. The values are averages of duplicate experiments.

Variation	Addition time	Rate immediately after addition, μl. O <sub>2</sub> per 10 min.	
		Control	Supplemented
Approximately 1 unit coenzyme A	min.		
0.2 ml. $2 \times 10^{-4}$ M cytochrome <i>c</i>	220	28	27
0.2 " 0.15 M citrate			28
0.2 " 0.15 " "			28
0.2 " 0.15 " "	200	31	29
0.3 " 0.01 M ATP	225	27	27
400 γ DPN, 20 γ TPN, 5 mg. NA			33
12% extract from boiled rat liver, 0.5 ml.			28
360 γ DPN, 5 mg. NA	275	18	27
30 " TPN, 5 mg. NA			19
360 " DPN, 30 γ TPN, 5 mg. NA			33
Cofactors*			28
" "	270	23	30

\* See Table III.

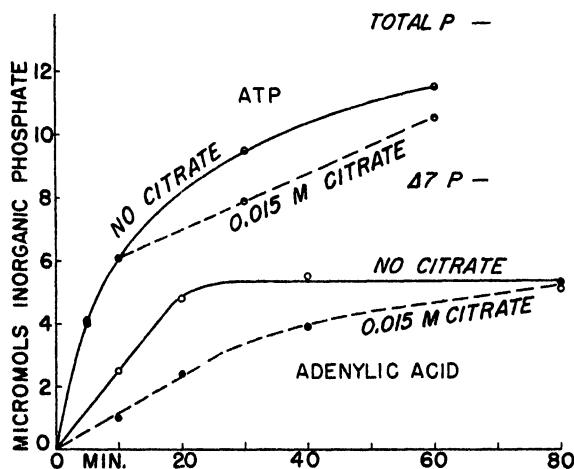


FIG. 3. Effect of citrate on ATP and adenylic acid breakdown. The standard conditions were used, except that phosphate was 0.0067 M and the homogenate had been incubated 25 minutes at 38° before use to destroy its oxidative ability. The pH was 7.3 to 7.4. The points are averages of duplicate determinations.

long duration, and this is probably in part due to the inhibitory action of citrate on adenylic acid breakdown (Fig. 3), indirectly preventing loss

of ATP. Citrate has been stated to be an inhibitor for ATPase (16) but this effect was not observed under our conditions.

A ratio of phosphate esterified per atom of oxygen used (P:O ratio) can be calculated as follows: with a part of the fresh homogenate used to obtain the data of Fig. 3, in a reaction mixture standard except that less phosphate was used so as to increase analytical accuracy, measurements showed no loss of ATP in the 1st hour (inorganic P at zero time  $21.9 \mu\text{M}$ , at 1 hour  $21.3 \mu\text{M}$ ). The rate of oxygen uptake at 1 hour was  $46 \mu\text{l.}$  per 10 minutes, or  $0.40$  microatom of oxygen per minute. From Fig. 3, the initial rate of ATP breakdown by incubated homogenate was  $1.0 \pm 0.1 \mu\text{M}$  of P per minute. If the rate of ATP breakdown by fresh homogenate was the same as by incubated homogenate, the rate of ATP synthesis in the reacting system must also have equaled  $1.0 \mu\text{M}$  of P per minute, and  $\text{P:O} = 1.0/0.4 = 2.5 \pm 0.3$ . A similar experiment agreed with the one cited. A recent estimate for the maximum P:O is  $2.8$  (17); therefore little oxidation occurred initially without concurrent phosphorylation.

### *Properties of Homogenate*

*Lability to Incubation*—Since other additions were not limiting, the homogenate was examined in view of earlier observations (1) on its lability. When a 10 per cent kidney homogenate in 8.5 per cent sucrose was incubated 10 to 15 minutes at  $38^\circ$ , it could no longer oxidize citrate, malate,  $\alpha$ -ketoglutarate, or oxalacetate, or reduce methylene blue in the reaction system. Since fresh homogenate in the complete reaction system is stable for several hours at  $38^\circ$ , the question arises as to what conditions are responsible for the difference in stability. It was found that the ATP present in the complete reaction system was the stabilizing factor, because homogenate in the reaction system minus only ATP was inactivated in 10 minutes at  $38^\circ$  and was not reactivated by later addition of ATP. If the reaction system lacked only substrate, the homogenate did not lose activity in 10 minutes at  $38^\circ$ , showing that ATP was of prime importance in preserving the homogenate. An adequate supply of ATP is necessary but not sufficient to preserve oxidation, because in the complete reaction system deterioration is either incompletely blocked by ATP or proceeds by a slower process which is not affected by ATP.

*Variable Amounts of Homogenate*—As can be seen from Table V, increasing the amount of fresh homogenate in the reaction system increased the initial rate of oxygen uptake proportionally and decreased the duration in an approximately linear manner. When incubated homogenate was added to the fresh homogenate, the initial rate rose from  $43$  to  $56 \mu\text{l.}$  in 10 minutes but was not further increased by addition of more incubated homogenate.

The stimulation by incubated homogenate was observed only at the beginning of the experiment and addition later in the experiment was ineffective. Thus, when 0.2 ml. of 10 per cent homogenate of rat kidney which had been incubated 25 minutes at 38° was added to a reaction system containing an equal amount of fresh homogenate, at zero time the rate was increased from 43 to 56  $\mu$ l. in 10 minutes. When the addition was made at 60 minutes, the rate was increased from 43 to 48  $\mu$ l., and when it was made at 240 minutes the rate increased only from 24 to 25  $\mu$ l. in 10 minutes. These results are interpreted to mean that fresh homogenate contains essential factors that are labile to incubation, which do not initially limit the rate because they are in slight excess. The

TABLE V

*Effect of Varying Amounts of Fresh and Incubated Homogenates*

The standard conditions were used except for the variable amounts of homogenate. The incubated homogenate was kept at 38° for 25 minutes prior to use. The values are averages of duplicate determinations.

Fresh homogenate	Incubated homogenate	Maximum rate	Duration (time to half maximum rate)
ml.	ml.	$\mu$ l. O <sub>2</sub> per 10 min.	min.
0.1		23	280
0.2		48	225
0.3		75	205
0.4		99	150
0.2		43	310
0.2	0.1	54	275
0.2	0.2	56	220
0.2	0.3	56	180
	0.2	5	

factor that limits the rate initially must be stable to incubation, because when incubated homogenate was added to fresh homogenate the rate increased. When the stable factor in the incubated homogenate was added in excess, a maximum rate of oxygen uptake was reached, which was determined by the amount of labile factors present in the fresh homogenate. After the reaction had progressed for a time, the labile factors were partly lost and were no longer in excess; so that addition of incubated homogenate was no longer helpful.<sup>4</sup>

<sup>4</sup> Our knowledge of the stable factors is limited because the assay is effective only within very narrow limits; the slight excess of labile material in whole homogenate permits only a small increase in oxygen uptake. Further work might be carried out with mitochondria as the source of labile factors. Although the mitochondria do not lack stable factors altogether, the large stimulation by incubated homogenate (in Table VII, among other data) indicates the superiority of mitochondria for assay of stable factors.

The dialyzable material from fresh or incubated homogenates was not stimulatory when added to fresh homogenate in the reaction system; therefore the rate-limiting stable factors were not dialyzable. They were present at the end of an experiment, after oxygen uptake ceased, as shown by the stimulatory effect when fresh homogenate was added. For example, in one experiment the initial rate was 48, the rate at 320 minutes was 4, and on addition of an amount of fresh homogenate equal to the original amount (and ATP) it rose to 62  $\mu$ l. in 10 minutes. ATP alone had no effect. No inhibitor that would have decreased the activity of the fresh homogenate was present, because the fresh homogenate was

TABLE VI  
*Bound DPN and Oxygen Uptake at Various Times*

The conditions were standard, except that 0.3 ml. of homogenate was used per flask. At the stated times the flask contents were removed from the Warburg apparatus, cooled on ice, and centrifuged for 10 minutes at  $8500 \times g$ . The supernatant fluid was discarded, and a small amount of water was added to the precipitate and heated 2 minutes at  $100^\circ$  to denature the enzymes. The assays were made without removing the solid material by a method in which a malic dehydrogenase system is employed (18). We wish to thank Dr. F. M. Huennekens for performing these assays.

Time	Rate	DPN per flask
min.	$\mu$ l. $O_2$ per 10 min.	$\gamma$
0		18
45	80	10
120	55	8
180	30	1
250	3	0

active when added to the inactive reaction mixture. Also, all essential components except for ATP and homogenate must still have been present.

*DPN in Homogenate*—Experiments by Huennekens and Green (18) have demonstrated that bound coenzymes in cyclophorase preparations are lost or destroyed under various experimental conditions. These authors suggest that the loss is an irreversible one, and that added DPN aids oxygen uptake by a different mechanism. The fact that addition of DPN during the course of the reaction provides a stimulation but does not stimulate initially in the present system suggests that bound DPN is lost as the reaction proceeds. At the suggestion of Dr. Green this was tested directly (Table VI). It can be seen that loss of DPN in the particles paralleled the decreasing rate of oxygen uptake.

*Centrifugal Fractionation*—Schneider and Potter (19) noted earlier that the mitochondria oxidized oxalacetate at a lower rate than did the whole homogenate, and that all fractions of the homogenate stimulated oxidation

TABLE VII

*Oxidation by Fractions of Homogenate*

The final concentrations were as follows: 0.067 M KCl, 0.017 M K phosphate (pH 7.0), 0.0033 M MgCl<sub>2</sub>,  $1.3 \times 10^{-5}$  M cytochrome c, 0.01 M potassium citrate, 0.008 M potassium pyruvate, 0.01 M potassium fumarate,  $1.3 \times 10^{-3}$  M K ATP. The final volume was 3.0 ml. The center well contained 0.2 ml. of 2 N NaOH. A 10 per cent homogenate of rat kidney in 8.5 per cent sucrose was used, and a portion was incubated at 38° for 20 minutes. The fractions, nuclei (N<sub>w</sub>), mitochondria (M<sub>w</sub>), and supernatant (S<sub>i</sub>) were prepared as described by Schneider and Potter (19). The duration is the time required for the rate to fall to one-half the maximum rate of the same flask.

Fraction of fresh homogenate	Incubated	Maximum rate <i>μl. O<sub>2</sub> per 10 min.</i>	Duration <i>min.</i>
0.2 ml. homogenate		50	195
0.4 " N <sub>w</sub>		3	
0.4 " M <sub>w</sub>		26	110
0.4 " S <sub>i</sub>		0	
0.4 " N <sub>w</sub>	0.2 ml. homogenate	9	70
0.4 " M <sub>w</sub>	0.2 " "	72	170
0.4 " S <sub>i</sub>	0.2 " "	14	50
0.2 " homogenate	0.4 " N <sub>w</sub>	60	185
0.2 " "	0.4 " M <sub>w</sub>	65	180
0.2 " "	0.4 " S <sub>i</sub>	61	100

TABLE VIII

*Oxygen Uptake by Various Tissues*

The standard conditions were used except for the type of tissue. The homogenates were 10 per cent rat tissue in 8.5 per cent sucrose except when otherwise noted. The numbers are averages of duplicate determinations.

Tissue	Homogenate	Maximum rate <i>μl. O<sub>2</sub> per 10 min.</i>	Duration (time to half maximum rate)
	<i>ml.</i>		<i>min.</i>
Kidney.....	0.2	45	250
" (H <sub>2</sub> O).....	0.2	30	100
Liver.....	0.6	47	370
" *(H <sub>2</sub> O).....	0.6	13	110
Heart.....	0.2	72	230
Brain.....	0.7	31	120
" .....	0.8	42	115
" .....	0.8†	44	135
Tumor (Flexner-Jobling).....	0.5	5	
" " (KCl) .....	0.5	4	
" (Walker No. 256) " .....	0.5	6	
" " " 256 " .....	0.5	4	

\* Conditions as in Table VII. 2 N NaOH in the center well.

† Plus cofactors (see Table III).

by the mitochondria, although the other fractions were inactive alone. By centrifugal fractionation of incubated and fresh homogenates and measurement of oxygen uptake by the various fractions alone and recombined, it was found that the necessary labile factors in the homogenate were in the mitochondria (Table VII), since only the mitochondria stimulated oxygen uptake by incubated whole homogenate. All incubated fractions contained some stable factor because they all stimulated fresh homogenate.

The mitochondria show gross damage when the homogenate is prepared in water rather than in isotonic solution (6) and this is correlated with reduced oxidation rate and duration (1). Observations of incubated homogenates under the phase microscope did not show such damage; the mitochondria looked more like those in a fresh homogenate, indicating that a more subtle breakdown process must have occurred.<sup>5</sup>

*Other Tissues*—The experiments described above were carried out with kidney homogenates. The maximum rate and duration for some other tissues are shown in Table VIII. Liver and heart were similar to kidney with respect to duration, but brain was inferior. An active DPNase in brain may be responsible (20), though the addition of cofactors and nicotinamide was only slightly helpful.

The stimulatory stable factors which are present in incubated homogenate are found in tissues which cannot take up oxygen by themselves in this system: Flexner-Jobling rat carcinoma, Walker 256 carcinosarcoma, and water homogenate of liver. These tissues stimulated oxygen uptake by fresh kidney homogenate to approximately the same extent that incubated kidney homogenate did, and reduced duration similarly.<sup>4</sup>

#### DISCUSSION

Throughout this communication the ability to take up oxygen has been considered a measure of the stability of both oxidative and phosphorylative enzymes. Although the enzymes and cofactors involved in the reactions resulting in oxygen uptake are of interest in themselves, their activity cannot be taken as evidence for the stability of enzymes and cofactors involved in other unmeasured reactions, *e.g.* synthetic processes. However, there is reason to believe that the stability of the oxidative processes, under the conditions of the present publication, are correlated with the stability of mechanisms which convert oxidative energy to high energy phosphate bonds. The fact that the P:O ratio had nearly the maximum possible value during the 1st hour of reaction shows that during this period, at least, little oxygen uptake was occurring without conversion of its energy to high energy phosphate bonds. Although it is possible that the mechanism for formation of high energy phosphate

<sup>5</sup> We wish to thank Dr. P. L. Gausewitz for carrying out these observations.

may have deteriorated preferentially, so that non-phosphorylative oxidation replaced phosphorylative oxidation, this seems unlikely from the data of Figs. 1 and 3, which show that not all of the ATP had broken down at the time oxygen was being utilized at half the maximum rate, and that, should phosphorylative oxidation have stopped, ATPase would have destroyed the ATP in a short time. This evidence does not prove that only phosphorylative oxidation occurred throughout the reaction, or that oxidation of certain substrates is inseparably coupled with phosphorylation, but it seems very likely that as long as oxygen uptake was one-half or more of the maximum rate phosphorylation was also occurring in this system.

Eventual failure of the system described in this communication is not due to loss of substrate, change of pH, production of inhibitor, or disappearance of ATP. In earlier work (1) loss of ATP was found to be correlated with failure of oxygen uptake. Although total loss of ATP will cause failure of the present system, if allowed to occur (by addition of 2,4-dinitrophenol, for example), the loss of ATP is diminished by high levels of citrate (0.015 M). Citrate decreases the rate of adenylic acid breakdown (perhaps by forming a complex with metal ions (14)), the effect being especially great at low adenylic acid levels, and this indirectly prevents the loss of ATP by providing a better opportunity for rephosphorylation of adenylic acid. The observed gradual breakdown of ATP probably results from deterioration of other compounds, which results in inability to synthesize high energy phosphate as fast as it is broken down by destructive enzymes. When oxygen uptake diminishes, the adenylic acid cannot all be rephosphorylated and the excess is broken down further, as shown by production of inorganic phosphate and ammonia (21).

The main conclusion that emerges from this study is that the failure of the system is due to loss of labile components in the mitochondria. These components were destroyed enzymatically, as shown by the fact that as the homogenate was increased the duration was decreased. In the present system loss of bound DPN is very likely a prime cause of failure of oxygen uptake, although significant losses of other bound coenzymes no doubt also occur. The view presented is similar to one recently published by Cross, Taggart, Covo, and Green (17), that the various steps of the Krebs tricarboxylic acid cycle require various protein-bound cofactors that are split off under unfavorable experimental conditions. Such an explanation could account for the dependence of duration on such factors as tonicity, ATP, citrate, cytochrome *c*, and the amount of homogenate present.

#### SUMMARY

A homogenate system which oxidizes the substrates of the Krebs cycle is described, in which the rate and duration are not limited by substrate,



buffering, or high energy phosphate. The system maintains high energy phosphate and oxygen uptake for 4 to 6 hours with rat kidney, heart, or liver as the enzyme source, but brain takes up oxygen for only 2 hours. Tumor is inactive but stimulates oxidation by kidney. The breakdown of the system was studied and it was concluded that the mitochondria lose cofactors and otherwise deteriorate. High energy phosphate is required for stability and its maintenance is aided by citrate, which inhibits the breakdown of adenylic acid that is continually formed from ATP, favoring resynthesis of ATP. A P:O ratio of  $2.5 \pm 0.3$  was calculated for the system for the 1st hour.

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## ISOLATION OF $\alpha$ -MONOPALMITIN FROM HOG PANCREAS\*

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In general, the glycerides which occur in nature are fully esterified; monoglycerides and diglycerides have only rarely been found. Bergmann (2) isolated 1,3-dipalmitin from commercial chrysalis oil but did not find it in freshly extracted chrysalises. Reichstein (3) and Wintersteiner and Piffner (4) identified  $\alpha$ -monopalmitin as a minor constituent of the adrenal lipides. Evidence (short of isolation) for the presence of monoglycerides in the intestine during the digestion of fats has been presented by Frazer and Sammons (5).

The present report describes the isolation, identification, and a simple method of preparation of highly purified  $\alpha$ -monopalmitin from hog pancreas tissue, in which it occurs in a remarkably high concentration. 1 to 1.2 per cent of the fresh weight of hog pancreas may be obtained in crystalline form, and analysis by a periodic acid method (6) indicates a total monoglyceride content amounting to approximately 10 per cent of the alcohol-soluble extractives.

*Isolation*— $\alpha$ -Monopalmitin was first isolated from hog pancreas by one of us (F. C. K.) while investigating methods of preparation of lipocaine from that source. The hashed fresh pancreas tissue was extracted several times with 65 per cent alcohol at pH 4 (HCl). The pooled aqueous alcohol extracts were concentrated under diminished pressure until essentially all the alcohol had been evaporated. The aqueous suspension remaining contained lipides which were eliminated by extraction with petroleum ether in a separatory funnel. When the layers separated, it was observed that a white, waxy, crystalline substance had appeared at the interface. After recrystallization from  $\text{CCl}_4$ , the substance was found to contain negligible amounts of N and P, and gave negative tests for S, carbohydrate, and cholesterol. Subsequently, larger quantities of the crude, crystalline ma-

\* A preliminary report was presented before the annual meeting of the American Society of Biological Chemists at Chicago, May, 1947 (1).

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terial were made available to us from pilot plant pancreas extracts prepared according to a similar procedure<sup>1</sup>.

*Identification*—The crude substance was purified by several recrystallizations from  $\text{CCl}_4$ , and found to be a saturated ester which, after saponification with alcoholic KOH, yielded palmitic acid and glycerol. Duplicate saponification-equivalent determinations were 328.0 and 328.5 (theory for monopalmitin, 330.5). The melting point was  $75.0\text{--}75.5^\circ$ ; mixed melting point with an authentic sample of synthetic  $\alpha$ -monopalmitin<sup>2</sup> (m.p.  $74\text{--}75^\circ$ ),  $74.3\text{--}75.5^\circ$ .  $\alpha$ -Monoglyceride assay by the periodic acid procedure of Pohle, Mehlenbacher, and Cook (6) yielded values of 99 to 100 per cent. Determinations in duplicate of free OH groups by the method of Ogg, Porter, and Willits (7) gave values of 10.2 and 10.45 per cent (theory, 10.3).

$\text{C}_{19}\text{H}_{38}\text{O}_4$ . Calculated.	C	69.04,	H	11.59
Found.	"	68.75,	"	11.22
	"	68.63,	"	11.24

18.18 gm. of purified ester were saponified with alcoholic KOH. After neutralization with dilute  $\text{H}_2\text{SO}_4$ , the alcohol was removed by distillation. The aqueous residue was acidified and the palmitic acid collected by filtration. It was washed repeatedly with water to remove glycerol and sulfate. The dry weight was 14.09 gm. or 77.5 per cent (theory, 77.5 per cent). After one recrystallization from acetone the melting point was  $61.7\text{--}62.6^\circ$ . The neutral equivalent values determined in triplicate were 254.9, 256.2, and 257.3; mean, 256.1 (theory, 256.4).

The filtrate and washings from the palmitic acid were dried by distillation under diminished pressure. The syrupy, salt-containing residue was extracted repeatedly with ethyl acetate and ethanol. The extracts were evaporated and again exhaustively extracted with absolute ethanol, the procedure being repeated several times to eliminate inorganic salt. The resulting light brown syrup, after desiccation, weighed 4.34 gm. or 23.9 per cent of the ester (theory, 27.9 per cent). It is assumed that part of the glycerol was lost during distillation of the alcohol and the subsequent manipulations. The isolated glycerol was redistilled under diminished pressure. The specific gravity of the product was 1.26 at  $22^\circ$ , and the refractive index at  $20^\circ$  was 1.4729, both these values agreeing precisely with those found in the literature.

The diacetate of the isolated  $\alpha$ -monopalmitin was prepared in pyridine solution by treatment with acetic anhydride. The resulting crystalline

<sup>1</sup> We are indebted to Dr. L. P. Anderson and Mr. Ralph Schmitt, Biochemical Pilot Plant, Armour Laboratories, for supplies of this material.

<sup>2</sup> Dr. H. C. Black, Research Laboratories, Swift and Company, Chicago, generously donated this sample.

product was dissolved in 10 parts of hot Skellysolve F, filtered while hot, and placed in the cold room at 3–5° for crystallization. The resulting needles were collected on a chilled Büchner funnel in the cold room. After thorough desiccation over  $P_2O_5$  they melted at 42.5–43.5°. Saponification-equivalent determinations in duplicate were 139.8 and 138.9 (theory for the diacetate, 139.5). No record of the previous preparation of the derivative has been found in the literature.

The dibenzoate was prepared by treating the ester in pyridine solution with benzoyl chloride. It was recrystallized and collected under the same conditions as the diacetate; m.p., 49.6–50.2°.

Further confirmation of the identity of the isolated  $\alpha$ -monopalmitin was provided by x-ray analysis of the purified ester carried out in the laboratories of The Procter and Gamble Company, Ivorydale, Ohio, through the courtesy of Dr. D. J. Kooyman.

TABLE I  
*Extraction of 500 Gm. of Hog Pancreas Tissue*

	Solvent volume	Total solids	$\alpha$ -Mono- glyceride
	ml.	gm.	per cent
First 3 alcohol extracts (filtered at room temperature) . .	3800	88.6	9.5
Second 3 " " ( " normal hot) . . . .	2250	22.0	0.8
One $CCl_4$ extract . . . . .	750	0.8	0

*Determination of  $\alpha$ -Monopalmitin Content of Hog Pancreas and Improved Method of Isolation*—Recognition that the isolation method described above was probably not an efficient one led us to further experiments.

500 gm. of ground hog pancreas were extracted by stirring mechanically with denatured alcohol at reflux temperature. The tissue was extracted six times with alcohol and once with  $CCl_4$ , each time for 3 hours. The first three alcohol extracts were filtered at room temperature; the fourth, fifth, and sixth were filtered while hot. The amounts of  $\alpha$ -monoglyceride (determined by the method of Pohle *et al.* (6)) and other extractives are shown in Table I. Although only 80 per cent of the total extractives was removed in the first three alcohol extracts, 98 per cent of the monoglyceride was found in this fraction. Other experiments confirmed these findings and furthermore showed that two alcohol extractions were sufficient to remove essentially all of the monoglyceride.

The procedure applied by Reichstein (3) to adrenal extracts was then followed (permutit treatment was omitted). In brief, the steps involved are removal of alcohol under diminished pressure, repeated extraction of the moist lipide residue with warm benzene, evaporation of the filtered

benzene extracts, extraction of the residue with hot acetone, and removal of insoluble phospholipide by filtration. Crystalline  $\alpha$ -monopalmitin may be obtained from the acetone filtrate in a variety of ways.

It was found that the benzene step could be omitted without disadvantage, but acetone treatment to eliminate phospholipides was essential. Attempts to crystallize the monoglyceride without removal of phospholipide were unproductive. Further simplification of the process by substituting acetone for alcohol as the original extracting solvent was not successful. Three acetone extracts removed only about 15 per cent of the monoglycerides present.

Additional experiments led to the following procedure for preparation of crystalline  $\alpha$ -monopalmitin from hog pancreas. (1) Extract ground hog pancreas tissue twice under a reflux, with stirring, with 2 liters of 95 per cent ethanol per kilo of wet tissue; (2) allow the digests to cool to room temperature and filter; (3) exhaustively evaporate the alcohol from the pooled extracts under diminished pressure; (4) allow the aqueous residue resulting from the alcohol evaporation to settle and separate the upper layer of semisolid lipid from the lower aqueous layer; (5) extract the moist lipid material with 5 volumes of hot acetone and filter while hot to remove the insoluble phospholipide; (6) exhaustively evaporate the acetone from the filtrate under diminished pressure; (7) heat the aqueous residue resulting from the acetone evaporation until the solids are dissolved or liquefied, cool to room temperature, separate the upper lipid layer from the lower aqueous layer, and dry the lipid material in a vacuum oven; (8) dissolve the dried lipids in 2.5 volumes of hot Skellysolve and allow to crystallize at room temperature. Collect the crystals by gravity filtration. Remove most of the rest of the solvent by pressure or suction filtration. Finally dry in a vacuum desiccator. Yield, 1.1 to 1.3 per cent of the fresh pancreas weight. Purity, 91 to 95 per cent by  $\alpha$ -monoglyceride assay.

For further purification recrystallize twice from 10 volumes of Skellysolve A, hexane, or heptane. A 90 to 95 per cent yield is obtained from each recrystallization, and the purity of the final product as estimated by  $\alpha$ -monoglyceride assay is 98 to 100 per cent. The melting point of one such product was 73.5–75° by the capillary method and 73–74° when the cooled molten solid was carefully heated in a well controlled air bath with the thermometer in the melting crystals.<sup>3</sup>  $\alpha$ -Monopalmitin exists in several polymorphic modifications, and the latter melting point compares with 74.6° found by Lutton and Jackson for the  $\beta'$  form (8).

In three experiments, residues, non-crystalline side fractions, and mother liquors were assayed for  $\alpha$ -monoglyceride content. The values obtained were added to the weight of crystalline  $\alpha$ -monopalmitin obtained to pro-

<sup>3</sup> Performed by Mr. Roger S. Sedgwick, Fat and Oil Research Section, Armour and Company.

vide an estimate of total  $\alpha$ -monoglyceride content. The results were 1.72, 1.77, and 1.9 per cent, respectively.

*Examination of Other Tissues*—The procedure described above for isolation of  $\alpha$ -monopalmitin was applied to brain and adrenal tissue of the hog in order to test the hypothesis that the pancreas is unique in its high content of  $\alpha$ -monopalmitin. No crystalline material was obtained in either case or from a liver lipide by-product.  $\alpha$ -Monoglyceride analysis of the fractions obtained indicated a content of not more than 0.11 per cent of fresh tissue weight in all three cases. Our failure to confirm the previous isolations of  $\alpha$ -monopalmitin from adrenal glands (3, 4) is not surprising, considering the small amounts of tissue extracted in our experiments and the small yields previously reported (0.004 per cent by Wintersteiner and Piffner).

#### DISCUSSION

The possibility that  $\alpha$ -monopalmitin is not a significant constituent of the living pancreas, but arises through postmortem enzymatic changes, was considered. It was found, however, that the same yield of crystalline substance was obtained when fresh pancreas taken directly from the killing floor was ground and promptly extracted as when stored frozen tissue or routinely handled pancreas was used as starting material. Furthermore, the method of isolation used is presumably not such as to cause either partial or complete hydrolysis of triglycerides.

We have no explanation for our failure to encounter other saturated monoglycerides as contaminants of the isolated monopalmitin. It is unlikely that the monoglyceride content indicated as present in the mother liquors, which in our hands resisted separation from the associated triglycerides, includes saturated monoglycerides other than monopalmitin, since their similar solubility and thermal properties would favor their precipitation under crystallization conditions. Unsaturated monoglycerides may possibly be present in this fraction, however.

The ease with which a high proportion of  $\alpha$ -monopalmitin may be isolated from pancreas encourages us to believe that by a combination of simple fractionations and periodic acid analysis a quantitative method for estimation of pancreas monoglyceride applicable to small laboratory animals is feasible. Such a method should facilitate investigations into the significance of its presence. It still remains to be demonstrated, however, that pancreas of other species is like that of the hog in its high  $\alpha$ -monoglyceride content.

#### SUMMARY

1. A white, waxy, crystalline substance isolated in high yield from alcohol extracts of fresh hog pancreas has been identified as  $\alpha$ -monopalmitin

by a variety of chemical and physical tests, including x-ray analysis, saponification to palmitic acid and glycerol, and preparation of derivatives.

2. A simple method of preparation has been developed which yields crystalline  $\alpha$ -monopalmitin to the extent of 1.1 to 1.3 per cent of the weight of fresh pancreas tissue.

3. The total  $\alpha$ -monoglyceride content of hog pancreas was estimated by a periodic acid assay procedure to be 1.7 to 1.9 per cent of the fresh tissue weight. In contrast, the  $\alpha$ -monoglyceride content of brain, adrenal, and liver was found to be 0.11 per cent or less.

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# STUDIES ON RIBONUCLEIC ACID

## II. METHODS OF ANALYSIS\*

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A complete knowledge of the composition of the nucleic acids has been unavailable because of the lack of quantitative procedures for the pyrimidine nucleotides. Our efforts to determine the composition of the ribonucleic acid (RNA) of pancreas resulted in the scheme of analysis outlined below. While this work was in progress methods were published for the separation and estimation of the purine and pyrimidine bases in hydrolysates of nucleic acid by paper chromatography (1, 2), the recovery of total nucleic acid nitrogen being incomplete (2). The procedure described here is sufficiently precise to account for 96 per cent (average recovery) of the nucleic acid nitrogen as individual purine bases and pyrimidine nucleotides.

The general plan of analysis includes hydrolysis by  $H_2SO_4$  with the liberation of free purines (3, 4), followed by separation of the purines as silver compounds in acid solution (5). From this filtrate, containing silver ions, the pyrimidine nucleotides may be precipitated quantitatively by making the solution alkaline and adding 3 volumes of isopropyl alcohol. By extraction of the two silver precipitates with hot HCl the purines and pyrimidine nucleotides are recovered separately and the individual components may be estimated by the spectrophotometric procedures described here. The pyrimidine nucleotides may also be estimated directly in the acid hydrolysate after removal of the purines without precipitation as silver compounds.

### EXPERIMENTAL

*Acid Hydrolysis of RNA*—The time interval required for acid hydrolysis of purine nucleotides without appreciable destruction of the pyrimidine nucleotides, previously reported by Jones (6) for yeast RNA with 5 per cent  $H_2SO_4$ , was determined for RNA of both yeast and pancreas with 2 N  $H_2SO_4$ . The preparations used were the acetic acid-insoluble fraction prepared from yeast RNA (7) and a specimen of pancreas RNA prepared by the methods described in the preceding paper of this series (8).

The liberation of inorganic phosphate at 100° from the more easily

\* Aided by a grant from the Rockefeller Foundation.

hydrolyzed purine nucleotides is complete in 60 minutes (Fig. 1). Inorganic and total phosphates were measured by the method of Fiske and Subbarow (9). The rate of liberation of purines was followed by precipitating the purines from the diluted hydrolysate with silver sulfate (5) and determining the total N in an HCl extract of the precipitate by the micro-Kjeldahl method (10). As shown in Fig. 1 the glucosidic union of purine with ribose-3-phosphate is broken with greater speed than the ester union of phosphate and ribose, the former being complete in 30 minutes or less.

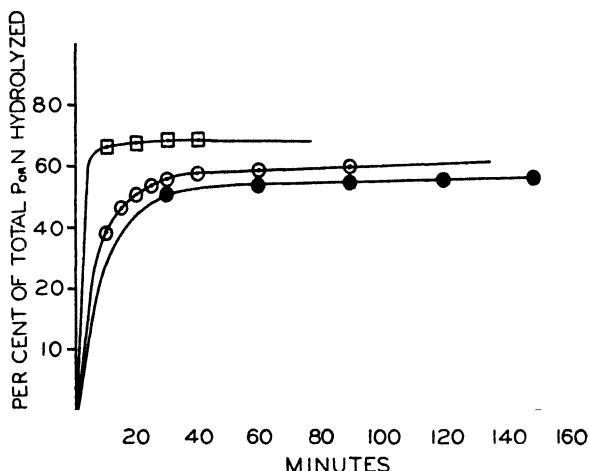


FIG. 1. Liberation of inorganic phosphate and of purine nitrogen from ribonucleic acid in 2 N  $\text{H}_2\text{SO}_4$  at  $100^\circ$ .  $\circ$  = P from yeast RNA (acetic acid precipitate);  $\bullet$  = P from pancreas RNA (acetic acid precipitate);  $\square$  = purine N from unfractionated pancreas RNA.

When purine-bound phosphate is to be measured, the hydrolysis should be continued for 1 hour, and the amount of inorganic P liberated should be corrected by the increase occurring during the next 30 minutes, determined on a second specimen (6). When only purines and the pyrimidine nucleotides are studied, the time of hydrolysis may be reduced to 30 minutes.

*Spectrophotometric Determination of Purines and Pyrimidine Nucleotides*—Ploeser and Loring (11) applied to the analysis of cytidine and uridine in mixtures a principle which we have followed in determining the purine and the pyrimidine nucleotides. The concentration of each component in a binary mixture can be calculated from the optical density measurements at two wave-lengths at which each component absorbs light to a different extent. The general formulae expressing the relationship of

the molar concentration  $C$  to the molecular absorption coefficients  $\epsilon$  and optical density  $D$  are as follows:

$$D_{\lambda_1} = C_a \epsilon_{\lambda_1}^a + C_b \epsilon_{\lambda_1}^b$$

$$D_{\lambda_2} = C_a \epsilon_{\lambda_2}^a + C_b \epsilon_{\lambda_2}^b$$

where  $D_{\lambda_1}$  and  $D_{\lambda_2}$  = optical densities at the two wave-lengths  $\lambda_1$  and  $\lambda_2$ ;  $C_a$  and  $C_b$  = concentrations in moles per liter of the two substances  $a$  and  $b$ ;  $\epsilon$  = molecular extinction coefficient, the subscript  $\lambda$  indicating the wave-length in  $m\mu$ , and the superscript, the compound (*e.g.*,  $\epsilon_{262}^a$  = molecular extinction coefficient for compound  $a$  at  $262 m\mu$ ).

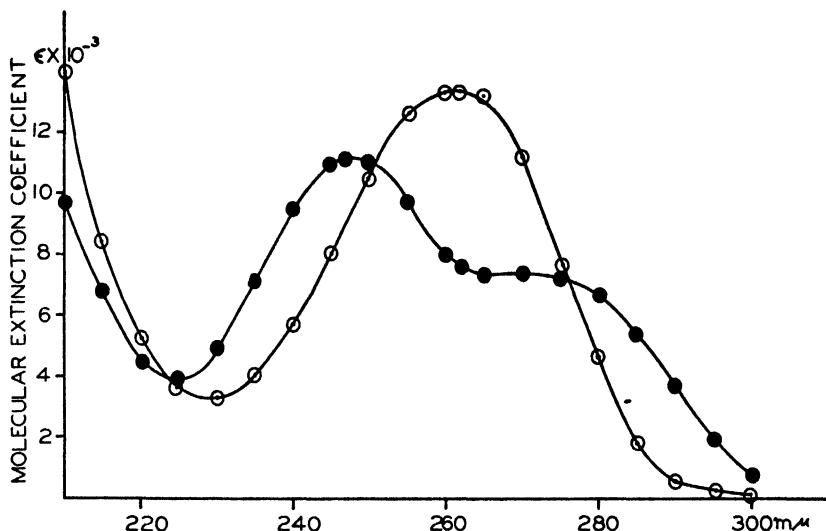


FIG. 2. Absorption curves of adenine and guanine in 0.1 N HCl. O = adenine sulfate (Eastman Kodak,  $6.02 \times 10^{-5}$  M); ● = guanine hydrochloride (Eastman Kodak,  $6.9 \times 10^{-5}$  M).

According to the plan of analysis proposed, adenine and guanine are obtained in the form of their hydrochlorides in N HCl solution, and the two pyrimidine nucleotides remain in the hydrolysate. The ultraviolet absorption curves for these two groups of compounds (Figs. 2 and 3) reveal sufficient differences in the molecular extinction coefficients at wave-lengths 245 and 262  $m\mu$  to permit calculation of adenine and guanine concentrations, while the maxima for cytidylic and uridylic acids (278 and 262  $m\mu$  respectively) appear to be suitable for the estimation of these nucleotides. The molecular absorption coefficients for these purines in 0.1 N HCl and for the two pyrimidine nucleotides in 0.01 N  $H_2SO_4$  are given in Table I. These values were obtained from measurements of op-

tical density on adenine sulfate and guanine hydrochloride (Eastman Kodak); cytidylic acid from Schwarz (2.99 N:1 P atoms), and a specimen prepared from yeast RNA by the method of Bredereck and Richter (12) (2.99 N:1 P atoms); and uridylic acid (1.95 N:1 P atoms) prepared by

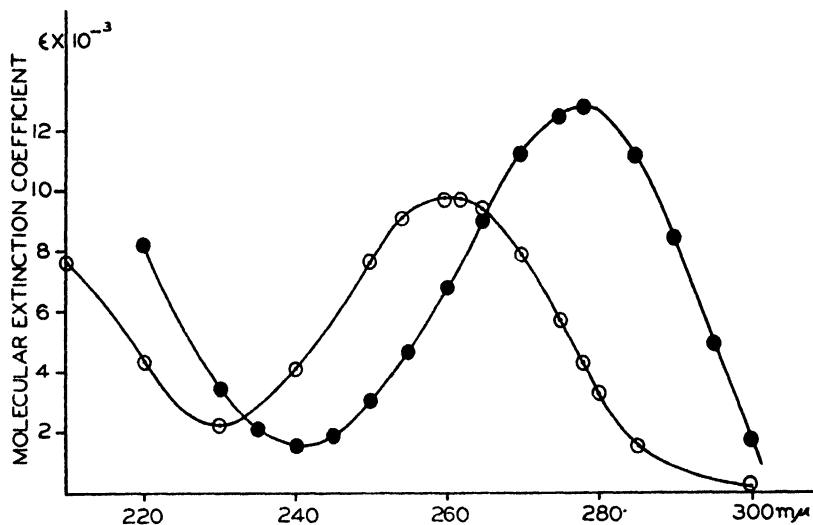


FIG. 3. Absorption curves of cytidylic and uridylic acids in 0.01 N HCl. ○ = uridylic acid,  $3375 \times 10^{-5}$  M; ● = cytidylic acid,  $285 \times 10^{-5}$  M.

TABLE I

*Molecular Extinction Coefficients of Adenine, Guanine, Cytidylic Acid, and Uridylic Acid*

Compound	Solvent	Molecular extinction coefficients				
		245 mμ	262 mμ	265 mμ	276 mμ	278 mμ
Adenine sulfate	0.1 N HCl	8,050	13,300		7100	
Guanine HCl	0.1 " "	10,900	7,580		7100	
Cytidylic acid	0.01 N H <sub>2</sub> SO <sub>4</sub>		7,880	9300		12,790
Uridylic " " "	0.01 " "		9,690	9300		4,300

deamination of cytidylic acid.<sup>1</sup> The concentration of the stock solutions examined was determined by measuring the nitrogen content (10). The measurements of optical density were made with the Beckman quartz spectrophotometer (model DU), quartz cells with a light path of 1 cm. being used.

Ploeser and Loring (11) reported molecular extinction coefficients of

<sup>1</sup> Kerr, S. E., and Doany, H., unpublished.

9890 for uridylic acid and 12,720 for cytidylic acid in 0.01 N HCl at the respective maxima 262 and 278  $m\mu$ . Heyroth and Loofbourow (13) found 10,300 at 260  $m\mu$  for adenine sulfate dissolved in water but state that Holiday<sup>2</sup> observed a maximum coefficient of 13,200 for a highly purified specimen of adenine sulfate. No data on the acidity of the solutions are given.

It should be noted that the molecular extinction coefficient for guanine differs with pH over a wide range (13, 14); hence it is important that optical density measurements be made always under the same conditions used for the determination of the coefficient  $\epsilon$ . The general formulae given above, when applied to solutions containing adenine (Ad) and guanine (Gu), become

$$D_{245} = C_{Ad} \epsilon_{245}^{Ad} + C_{Gu} \epsilon_{245}^{Gu}$$

$$D_{262} = C_{Ad} \epsilon_{262}^{Ad} + C_{Gu} \epsilon_{262}^{Gu}$$

When the values for  $\epsilon$  in 0.1 N HCl given in Table I are substituted in the above formulae and these are solved, the molar concentrations of adenine ( $C_{Ad}$ ) and guanine ( $C_{Gu}$ ) are expressed as follows:

$$(1) \quad C_{Ad} = \frac{10,900 D_{262} - 7580 D_{245}}{84.0 \times 10^6}$$

$$(2) \quad C_{Gu} = \frac{13,300 D_{245} - 8050 D_{262}}{84.0 \times 10^6}$$

In a similar manner formulae expressing the molar concentrations of cytidylic acid ( $C_C$ ) and uridylic acid ( $C_U$ ) were calculated and found to be the following:

$$(3) \quad C_C = \frac{9690 D_{278} - 4300 D_{262}}{89.9 \times 10^6}$$

$$(4) \quad C_U = \frac{12,790 D_{262} - 7880 D_{278}}{89.9 \times 10^6}$$

These formulae were tested on mixtures of adenine and guanine of known concentration, and likewise on mixtures of cytidylic and uridylic acids, with satisfactory results, as shown in Tables II and III.

Since the molecular extinction coefficients for adenine and guanine are identical at 276  $m\mu$  (the point of intersection of the two absorption curves), the sum of the molar concentrations of the two purines may be determined by dividing the optical density at 276  $m\mu$  by  $\epsilon_{276}$  or 7100. Likewise the

<sup>2</sup> Quoted by Heyroth and Loofbourow (13) as a personal communication from Holiday. This paper also reports corrections to the molecular extinction coefficients earlier given by Holiday (14).

sum of the molar concentrations of cytidylic and uridylic acids may be calculated by dividing the optical density at 265  $m\mu$  by the coefficient 9300 ( $\epsilon$  at the point of intersection of the curves for the two nucleotides). This method of estimating total purine and pyrimidine concentrations

TABLE II

*Analysis of Mixtures of Adenine and Guanine by Calculation from Optical Density at 245 and 262  $m\mu$  and Determination of Total Purine Concentration from Optical Density at 276  $m\mu$*

Taken, moles per liter, $\times 10^{-5}$			Found, moles per liter, $\times 10^{-5}$			Per cent recovery		
Adenine	Guanine	Total	Adenine	Guanine	Total*	Adenine	Guanine	Total*
3.42	2.26	5.68	3.32	2.35	5.77	97.2	104.1	103.2
1.71	2.26	3.97	1.64	2.27	4.04	95.8	100.6	101.8
3.42	1.13	4.55	3.34	1.17	4.56	97.7	103.5	100.1

\* Determined by dividing the optical density at 276  $m\mu$  by the molecular extinction coefficient 7100.

TABLE III

*Analysis of Mixtures of Cytidylic and Uridylic Acids by Calculation from Optical Density at 262 and 278  $m\mu$*

Taken		Found		Recovery	
Cytidylic	Uridylic	Cytidylic	Uridylic	Cytidylic	Uridylic
moles per l.	moles per l.	moles per l.	moles per l.	per cent	per cent
$0.255 \times 10^{-5}$	$0.519 \times 10^{-5}$	$0.247 \times 10^{-5}$	$0.500 \times 10^{-5}$	97.0	96.5
$0.510 \times 10^{-5}$	$0.260 \times 10^{-5}$	$0.501 \times 10^{-5}$	$0.202 \times 10^{-5}$	98.5	77.5
$1.423 \times 10^{-5}$	$3.375 \times 10^{-5}$	$1.416 \times 10^{-5}$	$3.486 \times 10^{-5}$	98.9	103.3
$3.99 \times 10^{-5}$	$4.48 \times 10^{-5}$	$3.89 \times 10^{-5}$	$4.49 \times 10^{-5}$	97.3	100.2
$7.98 \times 10^{-5}$	$4.48 \times 10^{-5}$	$7.89 \times 10^{-5}$	$4.63 \times 10^{-5}$	98.9	103.2
$5.80 \times 10^{-5}$	$8.95 \times 10^{-5}$	$5.80 \times 10^{-5}$	$9.00 \times 10^{-5}$	100.0	100.5
mg.		mg.			
0.933*		0.964		103.2	
1.260		1.235		98.0	

\* Precipitated by silver oxide in alkaline solution plus 3 volumes of alcohol, then extracted from silver precipitate with HCl.

provides a convenient check on the determinations of the individual constituents (see Table II).

Two procedures are described, one (A) to be followed when determinations of total N, total P, and purine-bound ribose are desired in addition to purine and pyrimidine nucleotides; the other (Procedure B) when only the concentrations of the purines and the pyrimidine nucleotides are sought.

*Procedure A*—The sample (about 50 mg. of RNA) is weighed into a test-tube ( $16 \times 150$  mm.), 5 cc. of 2 N  $H_2SO_4$  are added, and the tube is placed in boiling water for 30 minutes. The contents are agitated at the beginning to insure complete solution of the nucleic acid. After hydrolysis the cooled contents of the tube are diluted to 25 cc. in a volumetric flask. Aliquots of 1 cc. each are taken in duplicate for determinations of total P (9) and total N (10).

For the separation of purines from the pyrimidine nucleotides an 18 cc. aliquot is measured into a 25 cc. centrifuge tube with a conical tip, and 1 cc. of a molar suspension of silver oxide is added and stirred until dissolved. As soon as the silver purine precipitate has flocculated it is separated by centrifugation, the supernatant fluid being transferred to a 25 cc. volumetric flask. The precipitate is washed twice with 3 cc. portions of water, these being combined with the supernatant and diluted to 25 cc. This solution is reserved for the determination of pyrimidine nucleotides.

*Procedure B*—When only purines and pyrimidine nucleotides are to be determined, a 25 mg. sample of RNA is weighed into a 25 cc. conical tipped centrifuge tube, 4 cc. of 2 N  $H_2SO_4$  are added, the tube is placed in boiling water, and the sample is stirred until completely dissolved. After 30 minutes hydrolysis 12 cc. of water are added to reduce the acidity to 0.5 N, and 1 cc. of  $Ag_2O$  suspension is stirred into the entire hydrolysate. After cooling, the silver purine precipitate is separated by centrifugation and washed with two 3 cc. portions of water, the supernatant solution and washings being diluted to 25 cc. in a volumetric flask.

The silver purine precipitate and the acid solution (0.32 N) containing the pyrimidine nucleotides are analyzed as described below.

*Purine Determination*—The washed silver purine precipitate obtained by either procedure is extracted repeatedly with N HCl at  $100^\circ$ , the extracts being filtered into a 25 cc. volumetric flask and diluted with N HCl. 5 cc. of this solution, plus sufficient N HCl to make the final concentration exactly 0.1 N,<sup>3</sup> are diluted to 100 cc. with water for spectrophotometric examination. Optical density is measured at 245, 262, and 276  $m\mu$ . The blank solution used in the reference cell must be 0.1 N HCl treated rigidly in the same way as the unknown. The molar concentrations of adenine and guanine are obtained by calculation by Formulae 1 and 2 above. The sum of the molar concentrations of adenine and guanine is calculated by dividing the optical density at 276  $m\mu$  by 7100. Total N may be determined by micro-Kjeldahl in separate 2 cc. portions of

<sup>3</sup> The molecular extinction coefficient for guanine varies with acidity over a considerable range (13, 14). The coefficients for adenine and the pyrimidine nucleotides are the same in 0.1 N and 0.01 N solution; hence no adjustment of acidity need be made in the case of the pyrimidine nucleotides when the size of the aliquot is changed.

the HCl solution of the purines to confirm the spectrophotometric determinations.

*Pyrimidine Nucleotide Determination*—The acid solution containing the pyrimidine nucleotides and free of purines, obtained by either procedure, may be examined directly after suitable dilution in the spectrophotometer when pure specimens of nucleic acid are being analyzed, since the sulfuric acid, silver sulfate, and ribose phosphate present do not absorb light at the wave-lengths used.

For the spectrophotometric determination of cytidylic and uridylic acids a 5 cc. portion of the solution is diluted to 100 cc. with water<sup>3</sup> and the optical density is measured in the spectrophotometer at the wave-lengths 262, 265, and 278 m $\mu$ . The molar concentrations of cytidylic and uridylic acids are calculated by using Formulae 3 and 4 above. The total pyrimidine nucleotide concentration is calculated by dividing the optical density at 265 m $\mu$  by 9300. The result should be in close agreement with the sum of cytidylic and uridylic acid concentrations calculated from the optical densities. This sum, expressed in terms of N, should also agree with the total pyrimidine N as determined by the micro-Kjeldahl procedure in 5 cc. aliquots when protein-free nucleic acid is analyzed.

The data obtained from the spectrophotometric measurements are to be corrected for the various dilutions made and aliquots used, the final concentrations being expressed as mM per gm. or as per cent of the original RNA.

Occasions may arise in which the pyrimidine nucleotides cannot be measured directly by the spectrophotometric procedure, but only after separation from contaminating substances which absorb ultraviolet light. This may be accomplished by precipitation with silver in alkaline solution in the presence of isopropyl alcohol.

*Precipitation of Pyrimidine Nucleotides*—Cytidylic acid may be precipitated quantitatively under the conditions previously described for nucleosides (15), *i.e.*, when the solution containing silver ions is rendered alkaline to phenolphthalein. Uridylic acid is only partially precipitated by this procedure, but the addition of 3 volumes of isopropyl alcohol separates the remainder as an oil. The nucleotides are extracted from the washed silver precipitate with HCl, and may be determined spectrophotometrically in this extract. The satisfactory recovery of cytidylic and uridylic acid nitrogen by this procedure is shown in Table IV. In these experiments the pyrimidine nitrogen was determined by the micro-Kjeldahl procedure (8). Since many of the analyses to be reported in subsequent papers of this series were made by this procedure, it is described in some detail here.

*Procedure*—The acid solution containing the pyrimidine nucleotides,



freed from the purines by treatment with silver oxide, is transferred to a centrifuge bottle with a capacity of 100 cc. or more and made alkaline to phenolphthalein. 3 volumes of isopropyl alcohol are added with stirring and the mixture is centrifuged. The precipitate is washed twice with 75 per cent isopropyl alcohol, the supernatant fluid and washings being discarded. The precipitate is extracted with hot  $N$  HCl as in the procedure for purines, the filtered extracts being diluted to 25 cc. This extract serves for determinations of total pyrimidine  $N$  by the Kjeldahl method, and for optical density measurements as described above in the procedure for the pyrimidine nucleotides.

The alkaline silver precipitate contains not only the pyrimidine nucleotides, but also all of the organic and inorganic phosphate in the solution,

TABLE IV

*Precipitation of Cytidylic and Uridylic Acids with Silver Oxide and Isopropyl Alcohol*

Precipitant	N taken			N in $Ag_2O$ ppt.	Recovery
	Cytidylic	Uridylic	Total		
	mg.	mg.	mg.	mg.	per cent
$Ag_2O$ + 3 volumes ethyl alcohol.....	0.933			0.945	101.5
" + 3 " isopropyl alcohol....	1.26			1.26	100.0
" + 3 " ethyl alcohol.....		1.068		0.667	62.5
" + 5 " " ".....		0.734		0.515	70.0
" + 3 " isopropyl alcohol....		0.462		0.428	92.5
" + 3 " " ".....		0.196		0.220	112.0
" + 3 " " ".....	0.413	0.630	1.043	0.987	94.4
" + 3 " " ".....	0.288	0.630	0.918	0.925	100.5
" + 3 " " ".....	0.630	0.196	0.826	0.810	98.0

and some of the hydrolysis products of any protein which may have been present.

When the Procedure A described above was applied to pancreas RNA, 87 to 103 per cent (average 96 per cent) of the total  $N$  was accounted for as individual purines and pyrimidine nucleotides, the undetermined  $N$  being found in the acid hydrolysate after removal of the purines. Details of the analyses will be presented in a subsequent paper.

#### DISCUSSION

The procedures described above, giving directly the molar concentrations of each purine and pyrimidine nucleotide, obviate the need for such indirect approaches as the measurement of the rates of hydrolyzable to total phosphate (6, 4) as a means of determining the relative proportions of purine to pyrimidine nucleotide. These determinations may be useful,

however, in ascertaining whether each group of nucleosides is completely phosphorylated. Fletcher, Gulland, and Jordan (16) called attention to the fact that in some preparations of nucleic acid the percentage of phosphate is less than expected if each nucleoside were completely phosphorylated. When the molar concentration of total phosphate is less than the sum of the molar concentrations of the purines and the pyrimidine nucleotides, a determination of hydrolyzable (purine-bound) phosphate should indicate whether the deficiency is in the purine or the pyrimidine group of nucleosides. The amount of inorganic phosphate liberated in 60 minutes hydrolysis in 2 *N* H<sub>2</sub>SO<sub>4</sub> and corrected for the increase occurring in the next 30 minutes should correspond closely with the total purine and with ribose, each expressed as molar concentrations.

When the purine nucleosides are completely phosphorylated, there should be close correspondence between the molar concentrations of purine, ribose, and the inorganic phosphate liberated in 60 minutes hydrolysis, corrected for the increase occurring in the next 30 minutes. Likewise the concentration of the total pyrimidine nucleotides should correspond to pyrimidine-bound phosphate determined as the difference between total and hydrolyzable phosphate.

Ribose of the purine nucleotides may be determined in 0.1 cc. aliquots of the acid hydrolysate of nucleic acid by the colorimetric procedure (15), the period of heating with the orcinol reagent being increased to 45 minutes (17). The preliminary hydrolysis is unnecessary, since the orcinol reagent itself is strongly acid. We attempted to apply the method of Mas-sart and Hoste (18) to the determination of total ribose but were unable to secure reasonable results.

The scheme of analysis described in this paper calls for samples of 25 to 50 mg. of RNA. Since ample material was available, we have not yet experimented with a microprocedure, but it is obvious that the method may be modified to permit the analysis of much smaller samples, especially when only the spectrophotometric procedures are to be applied. In our procedure, only one-fifth of the HCl extracts are used for the optical density measurements, and these aliquots are diluted to 100 cc. An increase in the size of the aliquot and a reduction of the dilution should permit the satisfactory analysis of 1 mg. of material.

#### SUMMARY

A method is described for the analysis of ribonucleic acid. Provision is made for the separate measurement of purine nitrogen, pyrimidine nitrogen, hydrolyzable and total phosphates, purine-bound ribose, the individual purines, and the pyrimidine nucleotides. After separation of the purines as silver compounds from an acid hydrolysate of nucleic acid, the pyrimi-

dine nucleotides may be precipitated by silver in alkaline solution in the presence of isopropyl alcohol. Adenine, guanine, cytidylic acid, and uridylic acid are determined spectrophotometrically.

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## STUDIES ON RIBONUCLEIC ACID

### III. ON THE COMPOSITION OF THE RIBONUCLEIC ACID OF BEEF PANCREAS, WITH NOTES ON THE ACTION OF RIBONUCLEASE

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In a recent report on a procedure for the isolation of ribonucleic acid (RNA) from beef pancreas (1) it was shown that this compound is relatively labile, being partially depolymerized on incubation in solution at 25°, or when fractionated by precipitation with acetic acid (2, 3) or with a mixture of acetone and acetic acid (4). The widely varying composition reported in the literature for this compound (3, 5-10) may, consequently, be due to fragmentation of the native material during extraction and purification of the sample. A comparative study of the composition of RNA subjected to various methods of fractionation is, therefore, reported below.

Feulgen (11) was the first to report the presence of guanine in pancreas RNA in excess of that required for a tetranucleotide. Hammarsten (5), analyzing nucleoprotein prepared from pancreas with avoidance of both heat and alkalinity, found guanine and adenine in the ratio of 3:1, an amount of pentose consistent with a hexanucleotide, and purine nitrogen 75 per cent of the total. Jorpes (6), on the basis of determinations of total and acid-hydrolyzable phosphate, pentose, and total and purine nitrogen, concluded that the substance was a pentanucleotide. Isolation of the purine hydrochlorides from a suspension of the nucleic acid in methyl alcohol, however, revealed the guanine to adenine ratios in two experiments to be 3.3:1 and 4.6:1 (3). Jorpes (7) in a third attempt to solve this problem determined total purine before and after destruction of guanine, finding the guanine to adenine ratio to be 2:1, and concluded again that the pancreas ribonucleic acid was a pentonucleotide. Steudel (8), starting with Hammarsten's (12) procedure for preparation of the  $\beta$ -nucleoprotein, prepared the barium nucleinate according to Jorpes (6), converted this to the copper salt, and found it to contain 4 molecules of guanine to each of adenine nucleotide. He concluded that the RNA of pancreas contained five purine and two pyrimidine nucleotides. He had isolated only the cytidylic acid, but assumed uridylic acid to be present.

More recently Plentl and Schoenheimer (9), using the procedure of Levene and Jorpes (3), isolated the purines from the mixed nucleic acids of rat and beef pancreas, which they had extracted with hot 10 per cent NaCl.

The ratio of guanine to adenine in the RNA of rat pancreas was found to be 3:1, but that for beef pancreas was 0.55:1. Vischer and Chargaff (10) applied their chromatographic procedure to the RNA of pig pancreas, isolated by the method of Jorpes (6) and purified according to Levene and Jorpes (3) but still containing 3 per cent of desoxyribonucleic acid. They

TABLE I

*Analyses of RNA Prepared from Beef Pancreas and Fractionated by Various Procedures*

The results are expressed as mm per gm. of RNA (dried to constant weight *in vacuo* at 78°).

Experiment No.	RNA fraction	Spectrophotometric			Kjeldahl total purine	Spectrophotometric			Kjeldahl pyrimidine N	Cytidylic N plus uridylic N	Sum of nucleotides	Total P
		Guanine	Adenine	Total purine		Cytidylic acid	Uridylic acid	Total pyrimidine				
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
36-10A	Unfractionated*	1.06	0.25	1.31	1.29	0.79	0.24	1.19	4.17	2.85	2.34	2.44
36-10B	“ *	1.06	0.25	1.28	1.28	0.81	0.26	1.25	4.26	2.95	2.38	2.44
36-6	Non-dialyzable (cold)	1.02	0.43	1.46	1.44	0.87	0.38	1.37	3.65	3.37	2.70	2.68
36-4	“ “	1.05	0.38	1.59	1.43	0.87	0.36	1.22	3.95	3.33	2.66	2.66
36-15	Acetone-acetic acid ppt.	1.24	0.39	1.67	1.65	0.74	0.34	1.13	3.08	2.90	2.81	2.90
36-20A	“ “ *	1.16	0.42	1.56	1.44	0.85	0.35	1.33	3.50	3.25	2.78	2.75
36-20B	“ “ *	1.12	0.41	1.50	1.50	0.88	0.41	1.36	3.73	3.46	2.82	2.75
36-24	“ “	1.19	0.44	1.61	1.47	0.88	0.34	1.37	3.69	3.32	2.85	2.75
33-24	Acetic acid (83%) ppt.	1.12	0.29	1.41	1.41	0.88	0.36	1.24	4.22	3.36	2.65	
33-32	“ “	1.12	0.37	1.45	1.48	0.82	0.30	1.15	3.12	3.06	2.61	2.59
33-32	Alcohol ppt. in acetic supernatant	1.04	0.54	1.57	1.56	0.66	0.47	1.13	2.98	2.92	2.71	2.77

\* Experiments 36-10A and 36-20A hydrolyzed for 30 minutes, Experiments 36-10B and 36-20B hydrolyzed 60 minutes in 2 N H<sub>2</sub>SO<sub>4</sub>.

accounted for 82 per cent of the nitrogen, finding the nucleotides of guanine, adenine, cytosine, and uracil in the proportion of 2.42:1:1.23:0.3.

#### EXPERIMENTAL

The analyses presented in Table I were made on pancreas RNA prepared by the procedure recently described from this laboratory (1) and fractionated in several different ways. These included (a) removal of the low polymer fraction by dialysis, (b) precipitation with a mixture of 20 parts of acetone, 40 of acetic acid, and 40 of 0.14 M NaCl, (c) precipitation with 83 per cent acetic acid, followed by (d) precipitation from the supernatant solution with an equal volume of alcohol.

The analyses were made by the spectrophotometric procedure described in the preceding paper of this series (13), the results being expressed as molar concentrations in order to permit comparison of determinations which serve as checks. For example, total purine (Table I, Column 3) was determined by dividing the optical density at wave-length  $276\text{ m}\mu$  by the molecular extinction coefficient 7100, which at that wave-length is identical for adenine and guanine. The value thus obtained is in close agreement with the sum of the concentrations of adenine and guanine (Columns 1 and 2) as well as with the purine N determined by the micro-Kjeldahl procedure (Column 4). In the case of the pyrimidine nucleotides the sum of the concentrations given in Columns 5 and 6 does not agree so satisfactorily with total pyrimidine determined by optical density

TABLE II  
*Molar Ratio of Nucleotides in Pancreas RNA*

Experiment No.	RNA fraction	Guanine	Adenine	Cytidylic acid	Uridylic acid
36-10A	Unfractionated	4.41	1.04	3.30	1
36-10B	"	4.08	0.96	3.11	1
36-6	Non-dialyzable	2.68	1.13	2.29	1
36-4	"	2.91	1.05	2.42	1
36-15	Acetone-acetic acid ppt.	3.65	1.14	2.18	1
36-20A	" " "	3.31	1.20	2.43	1
36-20B	" " "	2.73	1.00	2.14	1
36-24	" " "	3.50	1.29	2.59	1
33-24	Acetic acid (83%) ppt.	3.11	0.81	2.44	1
33-32	" " (83%) "	3.73	1.23	2.73	1
33-32	Alcohol ppt. in acetic supernatant	2.21	1.15	1.40	1

measurement at  $265\text{ m}\mu$  (Column 7). In order to permit comparison of N determined by the Kjeldahl procedure after removal of the purines (Column 8) with the sum of N contained in cytidylic and uridylic acids, the values in Columns 5 and 6 were recalculated to represent mm of N rather than of nucleotides (Column 9). It is evident that an amount of N varying from a minimum of 0.18 to a maximum of 1.32 mm is found in the acid hydrolysate which cannot be accounted for as purine or pyrimidine.

The sum of the concentrations of adenine, guanine, cytidylic acid, and uridylic acid, each determined individually (Column 10), is in close agreement with the total phosphate (Column 11), the average difference being only 0.02 mm, or 1 per cent of the total.

The relative proportions of the four nucleotides are given in Table II, uridylic acid being taken as unity. In most of the specimens, adenine is

found in nearly the same concentration as uridylic acid. The molar ratio of the purines varies from 2.36 to 4.21 of guanine (average 3.5) to 1 of adenine. The pyrimidine nucleotides are found in ratios varying from 2.16 to 3.18 of cytidylic acid to 1 of uridylic acid.

#### DISCUSSION

Fletcher, Gulland, and Jordan (14) called attention to the low phosphorus content of some commercial preparations of yeast RNA, and suggested that a singly esterified phosphate might be lost from a nucleotide branching from the main nucleic acid structure. In our preparations, the close agreement between the molar concentrations of phosphate and the sum of the individual nucleotides indicates that each of the nucleosides is fully phosphorylated, regardless of whether the preparation was precipitated by alcohol, acetic acid, or the mixture of acetone, acetic acid, and NaCl.

The analytical procedures we employed account for 88 to 105 (average 97) per cent of the total nitrogen of the nucleic acid. Purine N determined by the Kjeldahl procedure is in close agreement with the sum of adenine and guanine N, any nitrogen unaccounted for being found in the hydrolysate after removal of the purines. Although our preparations have negative biuret tests, it is possible that the extra nitrogen represents the hydrolysis products of traces of protein. A qualitative test revealed the presence of some ammonium salts in the acid hydrolysate.

We are not satisfied that the hydrolysis of nucleic acid by 2 N  $\text{H}_2\text{SO}_4$  at 100° is without influence on cytidylic acid. We find that evaporation of cytidylic acid in N HCl solution to dryness at 100° in a stream of air results in the conversion of the major part of it to uridylic acid. A comparison of the analyses in Experiments 36-10A and 36-20B (Table I), however, reveals no loss of cytidylic acid when the hydrolysis period was increased from 30 to 60 minutes. The presence of ammonium salts in the hydrolysate may be of significance.

The variable ratio of guanine to adenine found is not surprising in view of the findings of others reviewed earlier in this paper. From the results summarized in Table II, it will be noted that the highest proportion of guanine (4.24:1) is found in the unfractionated specimens, and lower proportions in the preparations subjected to dialysis or to fractionation by means of acetic acid or acetone-acetic acid mixtures. The preparation containing the lowest proportion of guanine is that precipitated by alcohol after removal of the fraction insoluble in 83 per cent acetic acid. These variations may be attributed in part to alterations in the structure of the RNA during the course of its preparation or fractionation. The labile nature of the compound is indicated by its decomposition or depolymerization on incubation in aqueous solution (1).



Autolysis in the interval between death of the animal and fixation of the tissue must be considered as another factor causing variation in the ratios of guanine to adenine and cytidylic to uridylic acid. It is possible that RNA may also differ in composition in accordance with its participation in the metabolic processes of the cell. Investigation of the last two factors requires the development of quantitative procedures which may be applied to the tissue itself rather than to purified preparations of nucleic acid. A study of such procedures is in progress.

Our results are in harmony with the suggestion of Gulland (15) that the nucleotides may occur in random sequence in the polynucleotide.

*Action of Ribonuclease*—Since circumstances prevent the continuation of studies begun on the behavior of pancreas RNA with ribonuclease, the results of a typical experiment are presented in some detail below, together with a summarized statement of our general findings. It has already been noted (1) that, in aqueous solution, pancreas RNA is readily depolymerized at room temperature; hence an estimate of ribonuclease activity requires a careful comparison with control specimens incubated under the same conditions.

A specimen of pancreas RNA precipitated by a mixture of acetone, acetic acid, and NaCl, and analyzed for its content of purines and pyrimidines (13), was dissolved in water, adjusted to pH 5.3, and divided into two equal parts, each in a cellophane dialysis bag suspended in water at 25°. After 62 hours, the diffusion of phosphate having practically ceased, the dialysates were evaporated to a small volume in a vacuum and analyzed. To one of the bags containing the non-diffusible fraction crystalline ribonuclease (16) was added (0.8 mg. per 100 mg. of RNA) and the incubation of both control and enzyme-treated specimens was continued for another 24 hours. Only traces of phosphate were found in each dialysate. The non-diffusible enzyme-resistant fraction remaining in the bag, as well as the control, was then analyzed. In both specimens the RNA, although not diffusible, was no longer precipitable by 83 per cent acetic acid.

The results of analysis of the four fractions are given in Table III. Since no appreciable amount of phosphate diffused out after addition of the ribonuclease, it is evident that the non-diffusible fraction is resistant to the enzyme. Yeast RNA similarly treated behaved in an entirely different way. After the diffusible fraction had been removed by dialysis in the cold room, no further loss occurred when the dialysis was continued at 25°. Addition of ribonuclease at that point resulted in the diffusion of more than a third of the purine and more than half of the pyrimidine nucleotides. The hydrolytic effects of incubation of pancreas RNA thus resemble the enzymatic action of ribonuclease on yeast RNA. The addition of copper sulfate to the incubated RNA solution to a concentration of

0.02 per cent failed to inhibit this effect (17), which indicates that it cannot be attributed to the inclusion of Kunitz' enzyme in our preparation.

During incubation of pancreas RNA without added enzyme, the diffusion of the various nucleotides into the dialysate is not uniform, the loss of adenine exceeding that of guanine, with the result that the ratio of guanine to adenine becomes higher in the non-diffusible fraction. Uridylic acid also appears to diffuse to a greater extent than cytidylic acid. Other experiments, not recorded here, showed that incubation and dialysis of pancreas RNA resulted in an increase in the proportion of hydrolyzable

TABLE III

*Distribution of Purines and Pyrimidines after Incubation of Pancreas RNA with and without Added Ribonuclease*

Experiment No.	Specimen	Incubated and dialyzed  hrs.	Per cent of original nucleotide (molar ratios in parentheses)			
			Guanine	Adenine	Cytosine	Uracil
36-27	RNA (acetone-acetic acid ppt.)	0	100 (3.50)	100 (1.29)	100 (2.59)	100 (1)
36-27A	Control, diffusible	62	48.2 (3.80)	62.2 (2.06)	48.8 (2.88)	54.8 (1)
36-27A	“ non-diffusible	86	51.8 (4.93)	37.8 (1.52)	51.2 (3.64)	45.2 (1)
36-27B	Duplicate of Experiment 36-27A (no enzyme), diffusible	62	46.5 (2.43)	62.0 (1.33)	48.4 (1.73)	63.3 (1)
36-27B	Same + ribonuclease, non-diffusible	24	53.5 (4.83)	38.0 (1.41)	51.6 (3.19)	36.7 (1)
36-29A	Unfractionated RNA, control, non-diffusible	106	(4.58)	(1.06)	(3.16)	(1)
36-29B	Same + ribonuclease, non-diffusible	106	(3.84)	(1.49)	(2.46)	(1)

to total phosphate in the non-diffusible fraction. This is interpreted as evidence of the loss by diffusion of pyrimidine nucleotides to a greater extent than that of the purine nucleotides.

The material which diffuses into the dialysate may be fractionated by alcohol, a fifth of the phosphate being precipitated at 70 per cent concentration. No phosphate is split from the alcohol precipitate by the monophosphoesterase prepared from hypertrophic prostate tissue (18), and is presumably a low polymer nucleic acid. The remaining four-fifths of the diffusible fraction, after removal of alcohol by distillation in a vacuum, loses from 45 to 65 (average 55) per cent of its phosphate on treatment with the prostatic enzyme, which suggests that it may be a dinucleotide or a mixture of mononucleotides with nucleic acid of low molecular size.

That ribonuclease may hasten the effects also produced by long incubation without enzyme is revealed by the fact that, in short periods of dialysis (5 hours) at 25°, the enzyme greatly increased the fraction of nucleic acid rendered incapable of precipitation by uranyl acetate in 10 per cent trichloroacetic acid (16, 19).

Our observations confirm those reported by Schmidt (20) concerning the existence of a fraction of pancreas RNA resistant to the depolymerizing action of Kunitz' ribonuclease (16). In a personal communication, Schmidt states, however, that the RNA disappears almost completely within 3 hours during autolysis of a pancreas suspension at 37°, and suggests that the pancreas contains some other enzyme or factor which is necessary for the hydrolysis of the nucleic acid.

Ribonuclease has been used as a tool for differentiating between ribo- and desoxyribonucleoprotein in histochemical studies (21), disappearance of basophilia after treatment of a tissue with the enzyme being considered evidence of the presence of ribonucleic acid. In view of the fact that a fraction of RNA is resistant to the action of this enzyme, caution must be used in the interpretation of histochemical findings based on its use.

#### SUMMARY

Analyses are reported for individual purines and the pyrimidine nucleotides in pancreas RNA. These account for all of the phosphorus and for 97 per cent (average) of the total nitrogen.

The molar ratios of the purines vary from 2.36 to 4.21 molecules of guanine to 1 of adenine. The pyrimidine ratios vary from 2.16 to 3.18 molecules of cytidylic acid to 1 of uridylic acid. Adenine and uridylic acid are found in nearly equal molar concentrations.

The composition of the RNA appears to be dependent partly on the mode of preparation. After removal of diffusible material by dialysis in the cold, further incubation at 25° causes extensive depolymerization of pancreas RNA (but not of yeast RNA), adenine and uracil nucleotides being lost by diffusion to a greater extent than the others. Treatment of the non-diffusible residue with ribonuclease results in the production of no new diffusible fragments.

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# INHIBITORY EFFECT OF MUSCLE ADENYLIC ACID ON ANAEROBIC GLYCOLYSIS OF BRAIN\*

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In the course of studies on the biosynthesis of nucleotides it was observed that adenosine-5-phosphoric acid (AMP-5) and inosine-5-phosphoric acid (IMP-5) inhibited the lactic acid production of brain homogenates. This finding was unexpected, since AMP-5 is known to accept phosphate from phosphopyruvic acid (1, 2) and would be expected under the proper conditions to promote glycolysis. Since AMP-5 is a natural substance, the possibility exists that this inhibitory effect normally is involved in controlling the rate of glycolysis.

This paper records experiments bearing on the specificity and the degree of this inhibition in mouse and pig brain preparations.

## EXPERIMENTAL

The earlier studies were made with mouse brain homogenates. Later for convenience an acetone powder of swine brain was employed. Swine brain provided an acetone powder which was more active than that of sheep or cattle and maintained its glycolytic activity for several months when kept at 5° in a desiccator under a vacuum. Fresh swine brain was obtained on ice from the local slaughter-house approximately 2 hours after removal, and without delay the acetone powder was prepared in the cold room at -10°. 1 part of brain was homogenized for 3 minutes with 10 parts of -10° acetone in a Waring blender, filtered through coarse paper with suction, and washed on the paper with additional acetone. The precipitate was resuspended in 10 volumes of cold acetone and rehomogenized 1 minute. After filtering, washing, and partially drying by suction, the powder was dried over sulfuric acid *in vacuo* at 5°. In the present study three preparations of such an acetone powder were employed.

Lactic acid formation was measured by carbon dioxide evolution in Warburg vessels. No correction for retention of CO<sub>2</sub> was made. In some experiments lactic acid also was determined in CdSO<sub>4</sub>-NaOH filtrates by the colorimetric method of Barker and Summerson (3). Glycolysis was measured essentially as described by Utter,<sup>1</sup> who has been able to obtain

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<sup>1</sup> Dr. M. F. Utter, personal communication.

good levels of glycolysis in mouse and rat brain homogenates without the addition of hexose diphosphate (HDP). Since the inhibitory effect of AMP-5 is greatly diminished in the presence of HDP, his procedure has facilitated the present observations.

The reaction system generally was prepared as follows. The main compartment of the flask contained 0.15 ml. of 0.16 M  $\text{KHCO}_3$ , pH 7.8, 0.5 ml. of a combined solution<sup>2</sup> of adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), nicotinic acid amide, glucose, and  $\text{MgCl}_2$ , and also AMP-5 or other compounds at the indicated concentrations. In the side arm of each vessel were placed 0.05 ml. of 0.16 M  $\text{KHCO}_3$ , pH 7.8, 0.05 ml. of 0.1 M  $\text{K-PO}_4$  buffer, pH 7.4, and 0.15 ml. of homogenized swine brain acetone powder (30 to 45 mg. per ml. of water). The total volume was 1.1 to 1.2 ml. The homogenate was prepared in an ice bath and used immediately. All solutions were neutralized to approximately pH 7 to 7.5 with KOH, with brom thymol blue as an internal indicator. Care was exercised to maintain an equality of salt concentrations in the control and test vessels, since it was observed occasionally that an excess of potassium ion produced a slight inhibition. Thus KCl was added to all control vessels in an amount equivalent to the quantity of KOH required to neutralize the test substances. The vessels were gassed 5 minutes at room temperature with 95 per cent  $\text{N}_2$ -5 per cent  $\text{CO}_2$ . The reaction temperature was 38°. The contents of the side arms were tipped immediately and an equilibration period of 10 minutes was allowed before the zero time readings were taken.

Unless otherwise stated, a commercial muscle adenylic acid was<sup>3</sup> employed. In some experiments preparations of AMP-5 made by a modification of the procedure of Kerr (4) were used; one of these was prepared by Dr. M. F. Utter, the other by the author. Finally comparison was made with a pure preparation of AMP-5 from the laboratory of Parnas available in this department. Inosine-5-phosphoric acid was prepared enzymatically from muscle adenylic acid by the technique of Kalckar (5) and purified as the barium salt. Adenosine triphosphate was prepared by a method similar to that of Dounce and coworkers (6). Diphosphopyridine nucleotide was obtained by the procedure of Williamson and Green (7). Adenosine and yeast adenylic acid were commercial preparations. HDP was prepared as the potassium salt from the commercial barium salt.

<sup>2</sup> This solution consisted of the following proportions: 0.005 M ATP, 3.0 ml.; 0.30 M glucose, 1.2 ml.; 0.08 M  $\text{MgCl}_2$ , 0.6 ml.; 7 mg. of DPN and 25 mg. of nicotinamide in 1 ml. of water, 1.2 ml. The DPN and nicotinamide were dissolved just before use.

<sup>3</sup> Bischoff adenosine-5-phosphoric acid. The author wishes to express his gratitude to the Ernst Bischoff Company, Inc., for this gift.

### Results

A typical experiment showing the inhibition of lactic acid production by adenosine-5-phosphoric acid is presented in Table I. In Tables I to VI the values are the averages from two vessels. Similar results have been obtained with both mouse brain homogenates and pig brain acetone powder preparations and are representative of numerous observations. It has been observed that the percentage inhibition of AMP-5 generally is greater, the slower the initial rate of glycolysis. When the rate of gly-

TABLE I

*Effect of Muscle Adenylic Acid on Lactic Acid Formation in Swine Brain Acetone Powder*

Time  min.	AMP-5 molarity				
	Control	$0.7 \times 10^{-3}$	$1.4 \times 10^{-3}$	$2.8 \times 10^{-3}$	$4.2 \times 10^{-3}$
	$\mu\text{l. CO}_2$	Per cent inhibition			
0-20	94	25	35	57	66
20-40	103	24	37	66	80
40-60	95	1	26	59	83

TABLE II

*Comparison of Inhibition by Adenosine-5-phosphoric Acid of Lactic Acid Production and CO<sub>2</sub> Evolution*

Measurement of glycolysis	Control	Plus 0.0031 M AMP-5
	$\mu\text{M}$	$\mu\text{M}$
CO <sub>2</sub> evolution.....	7.90*	3.85*
Lactic acid.....	8.42	4.10

Reaction time, 50 minutes.

\* Extrapolated to zero time from 10 to 50 minute CO<sub>2</sub> evolution values.

colysis is high, or the concentration of AMP-5 low, and thus the degree of inhibition low, the inhibition frequently tends to disappear with time. On the other hand a marked inhibition normally becomes greater with time.

The inhibitory effect of AMP-5 is on the lactic acid production, as shown by the experiment in Table II. Approximate agreement exists between the CO<sub>2</sub> evolution values and the actual lactic acid production as measured by the colorimetric method.

The specificity of this inhibition is shown in Table III. It may be observed that the presence of phosphate on the 5 position of ribose in the

nucleotide causes an inhibition, whereas at the indicated concentrations the presence of phosphate on position 3 is without effect. This same specificity has been found with mouse brain homogenates. The fact that ATP did not inhibit glycolysis, whereas AMP-5 prepared from the same ATP by  $\text{Ba}(\text{OH})_2$  hydrolysis did, suggests that the inhibitory effect

TABLE III

*Specificity of Inhibition of Lactic Acid Production by Adenosine-5-phosphoric Acid*

Time	Control	Commercial AMP-5	Parnas AMP-5	Yeast AMP (AMP-3)	ATP	Adenosine
		$1.39 \times 10^{-3} \text{ M}$	$1.50 \times 10^{-3} \text{ M}$	$1.44 \times 10^{-3} \text{ M}$	$2.44 \times 10^{-3} \text{ M}$	$1.50 \times 10^{-3} \text{ M}$
	$\mu\text{l. CO}_2$	Per cent inhibition				
min.						
20-65	70	44	39	3	5	5
65-107	78	38	30	6	14	8

Includes the  $0.001 \text{ M}$  ATP added to all the flasks.

TABLE IV

*Inhibition of Lactic Acid Production by Different Preparations of AMP-5*

Time	Control	Preparation A	Preparation B	Preparation C
	$\mu\text{l. CO}_2$			
min.				
10-40	108	108	119	109
AMP added at 42 min.	—	+	+	+
	$\mu\text{l. CO}_2$	Per cent inhibition		
50-80	110	23	27	36

Final concentration of AMP-5,  $1.66 \times 10^{-3} \text{ M}$ . Preparation A, Bischoff; Preparation B, by the author; Preparation C, by Dr. M. F. Utter. Reaction mixture, the same as for the other experiments with the exception that 0.15 ml. of 1:10 mouse brain homogenate in water was used.

is not due to impurities preexisting in ATP and carried over into AMP during preparation of the latter, although the hydrolysis could have produced an inhibitor.

Table IV compares the inhibition of glycolysis obtained with different preparations of AMP-5. It will be observed that in general these preparations showed a similar degree of inhibition. In Table III, it is found that the commercial AMP-5, which is Preparation A of Table IV, inhibits glycolysis to approximately the same degree as AMP-5 from the laboratories



of Parnas. From an analytical point of view these AMP-5 preparations were highly purified, but it would be presumptuous to claim that they did not contain even traces of impurity. However, it must be argued that in all probability the quantity of impurity would vary from one to another because of the difference in preparation and source. Therefore the general similarity in the extent of inhibition caused by these substances suggests that the inhibition is not due to an impurity.

TABLE V  
*Greater Inhibition of Glycolysis by Inosine-5-phosphoric Acid Than by AMP-5*

Time	Control	AMP added		IMP added	
		$1.20 \times 10^{-3} \text{ M}$	$0.6 \times 10^{-3} \text{ M}$	$1.30 \times 10^{-3} \text{ M}$	$0.65 \times 10^{-3} \text{ M}$
	$\mu\text{l. CO}_2$	Per cent inhibition			
min.					
10-40	122	21	10	63	27
40-60	83	22	22	83	47

TABLE VI  
*Effect of Added Hexose Diphosphate on Inhibitory Action of Adenosine-5-phosphoric Acid*

No HDP			Plus $4.1 \times 10^{-3} \text{ M}$ HDP		
Time	Control	AMP $1.4 \times 10^{-3} \text{ M}$	Control	AMP $1.4 \times 10^{-3} \text{ M}$	AMP $2.8 \times 10^{-3} \text{ M}$
(1)	(2)	(3)	(4)	(5)	(6)
min.	$\mu\text{l. CO}_2$	per cent inhibition	$\mu\text{l. CO}_2$		per cent inhibition
0-10	39	23	60	13	35
10-25	48	33	95	4	9
25-62	125	46	151*	-11	-10

\*Not linear at this point.

It is pertinent to point out that Utter<sup>1</sup> has found that the AMP-5 Preparation A of Table IV is capable of being phosphorylated by the pyruvate transphosphorylase system of mouse brain. Thus while AMP effects an inhibition in one part of the glycolytic system, it is capable of accepting phosphate in another.

In Table V is compared the inhibition of glycolysis caused by AMP-5 and IMP-5. From these data it is apparent that IMP-5 tends to produce the greater inhibition.

Table VI shows the effect of added hexose diphosphate in the reaction mixture. Occasionally the addition of HDP to these systems produces an enhanced rate of glycolysis. Therefore, in Table VI, Column 3 should be compared with Column 2, and Columns 5 and 6 should be compared with

Column 4. It is apparent that the presence of HDP overcomes the inhibitory effect of AMP-5. However, a definite inhibition is present at the first 10 minute measurement which, because of the 10 minute equilibrium period, actually represents the period 10 to 20 minutes after the reaction started. The addition of HDP after the inhibition has been established has been found to speed the return to the control rate. In experiments in which HDP was the sole substrate the AMP inhibition occurred only in the first 10 to 20 minutes.

#### DISCUSSION

It is generally considered that, among other factors, the level of ATP is important in controlling the rate of anaerobic glycolysis. In view of the inhibitory effect of adenosine-5-phosphoric acid, which is the product of adenosinepyrophosphatase activity on ATP, the balance between the reactions forming and removing AMP-5 perhaps should be considered as an additional factor controlling the glycolytic rate.

The inhibitory effect of AMP-5 on anaerobic glycolysis of brain is somewhat surprising, since this nucleotide has been shown by Parnas and his coworkers (1) and by Lohmann and Meyerhof (2) to accept phosphate from phosphopyruvic acid in muscle extracts and by Utter<sup>1</sup> in brain preparations. Thus on the one hand AMP-5 promotes and on the other hand inhibits glycolysis. This raises the question as to how AMP-5 is concerned in normal glycolysis. It is conceivable that ordinarily the level of AMP-5 is low and that the usual acceptor of phosphate in this reaction is adenosine diphosphate rather than adenosine monophosphate (8).

It is apparent that the inhibition exhibited by AMP-5 frequently disappears with continuing glycolysis. It may not be clear why the inhibition should disappear in one instance and not in another under apparently identical conditions. However, it may be pointed out that this glycolytic system is rather finely balanced in regard to the phosphorylation and dephosphorylation reactions, and small differences in conditions may cause the system to be driven in one or the other direction. Since it has been observed that AMP-5 is phosphorylated by brain phosphoenol transphosphorylase,<sup>1</sup> it is reasonable to assume that this reaction is concerned in removing the AMP-5. When glycolysis is slow and this phosphorylation reaction is retarded accordingly, the AMP-5 effect usually becomes more pronounced, and this in turn could result in further breakdown of ATP by adenosinepyrophosphatase forming additional AMP-5. Thus the AMP-5 effect is cumulative. On the other hand, when the phosphorylation reaction is rapid, the AMP-5 is phosphorylated and the inhibition disappears. In this regard the effect of HDP in overcoming the inhibition may be due

to formation of a large excess of phosphopyruvate for the phosphorylation. In addition the greater inhibitory action exhibited by IMP-5 as compared to AMP-5 may arise from the possibility that the former is not phosphorylated, there being no available evidence of such a reaction. Both of these nucleotides also are degraded by brain homogenates to non-inhibitory compounds at a relatively slow rate,<sup>4</sup> perhaps by the reactions reported by Dische (9) and Schlenk and Waldvogel (10) for such compounds.

It is to be noted that the concentrations of AMP-5 required to bring about this inhibition are of the order of  $10^{-3}$  M and that such concentrations do not normally exist in the brain. However, it should be recognized that in general in *in vitro* systems it is necessary to add metabolites and cofactors at high concentration to demonstrate their action. For example ATP is added at a concentration of  $10^{-3}$  M. It is not unreasonable to expect that a competition between AMP-5 and a cofactor for an enzyme surface might require concentrations of AMP-5 comparable to those of the cofactor.

In the course of an investigation of malic dehydrogenase, Green (11) observed that both AMP-5 and ATP inhibited the system and suggested that this effect was due to a competition with diphosphopyridine nucleotide for the enzyme as a result of the partial structural similarity between these compounds. Whether an analogous explanation would apply to the inhibition of anaerobic glycolysis by AMP-5 must await further experimental evidence.

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#### SUMMARY

Adenosine-5-phosphoric acid and inosine-5-phosphoric acid inhibit anaerobic glycolysis in brain preparations. This inhibition is not produced by adenosine, adenosine-3-phosphoric acid, and adenosine triphosphate.

The inhibitory effect of AMP-5 generally decreases as glycolysis proceeds when the initial inhibition is not great. Hexose diphosphate added to the reaction system rapidly overcomes the inhibition by muscle adenylic acid.

The possible significance of this inhibition is discussed.

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# STRUCTURAL REQUIREMENTS OF SPECIFIC SUBSTRATES FOR CARBOXYPEPTIDASE\*

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Typical peptide substrates for carboxypeptidase are *N*-acylglycyl derivatives of phenylalanine, tyrosine, tryptophan, and leucine (1-8), the susceptibility of the peptides to hydrolysis decreasing in the order of amino acids named.<sup>1</sup> These compounds contain in addition to the hydrolyzable bond a secondary peptide bond which is separated from the former by one methylene group. The importance of this bond is evidenced by the finding that its substitution by a terminal amino group, as in glycyl-L-tyrosine (2), or its spacial separation from the susceptible bond by one additional methylene group (carbobenzoxy- $\beta$ -alanyl-L-phenylalanine (9)) greatly diminishes the susceptibility to hydrolysis by carboxypeptidase. However, substrates devoid of both a positively charged amino group and a secondary peptide bond have long been known to be hydrolyzed by this enzyme. Thus chloroacetyl-L-tyrosine and its phenylalanine analogue were one of the earliest substrates used to test the enzymatic activity of carboxypeptidase (2, 5, 10).

In the present investigation an attempt was made to evaluate the contribution of the secondary peptide bond of specific peptide and ester (11) substrates to hydrolysis by this peptidase. The quantitative kinetic measurements were interpreted in terms of the two-step reaction mechanism previously applied to other enzymatic systems (8, 12) in order to evaluate the effect of structural modification of the substrates on both the Michaelis constant,  $K_m$ , and the specific activation rate,  $k'$ . As a part of this study, the influence of temperature on the rate of activation has been determined for one of the present substrates.

\* Part of a thesis submitted by J. E. Snoke to the Graduate School of Duke University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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<sup>1</sup> Although the affinity of carbobenzoxyglycyl-L-tryptophan for carboxypeptidase seems to be higher than for the corresponding derivative of phenylalanine, as evidenced by apparent adherence to zero order kinetics (6), the specific rate of hydrolysis,  $k'$ , of the former is appreciably lower. The hydrolysis of tryptophan derivatives by carboxypeptidase was first described by Hofmann and Bergmann (2).

## EXPERIMENTAL

*Enzyme*—Carboxypeptidase recrystallized six times was prepared by Dr. E. Elkins-Kaufman of this laboratory (13).

*Substrates*

*Hippuryl-DL-phenylalanine (HipP)* was prepared by coupling benzoyl-glycine azide with DL-phenylalanine (14). M.p., found, 176–177°; reported (14), 172°.

Calculated, N 8.58; found, N 8.56

*Chloroacetyl-DL-phenylalanine (ClAcP)*—DL-Phenylalanine was acylated with chloroacetyl chloride (15). After repeated recrystallizations, the melting point was 108–109°; reported (15), 131°.

Calculated, N 5.78; found, N 5.79

*Chloroacetyl-dl-β-phenyllactic Acid (ClAcPLA)*—A mixture of 5.0 gm. of dl-β-phenyllactic acid (16) and 13 gm. of chloroacetyl chloride was heated on a steam bath under anhydrous conditions for 1 hour and the excess acid chloride was removed by concentration *in vacuo* (70° at 25 mm. pressure). The remaining yellow oil was taken up in ether, thoroughly washed with water, and then, after drying over anhydrous Na<sub>2</sub>SO<sub>4</sub>, the ether was removed by concentration *in vacuo*. The product resisted all attempts of crystallization and had a neutralization equivalent of 248; calculated, 242.5.

*Bromoacetyl-dl-β-phenyllactic Acid (BrAcPLA)*—Phenyllactic acid was acylated with bromoacetyl bromide as described above for ClAcPLA and the product purified by several extractions with hot water. The product could not be crystallized. Neutralization equivalent 289; calculated, 287.

*Acetyl-DL-phenylalanine (AcP)* was prepared according to du Vigneaud and Meyer (17). M.p., found, 146–147°; reported (17), 150–151°

Calculated, N 6.76; found, 6.71

*Acetyl-dl-β-phenyllactic Acid (AcPLA)*—DL-β-Phenyllactic acid was acylated with acetyl chloride (18). M.p., found, 65–67°; reported (18), 72°; neutralization equivalent, 212; calculated, 208.

*Benzenesulfonylglycyl-DL-phenylalanine (BSGP)*—To a suspension of 10 gm. of powdered, dry benzenesulfonylglycine in 100 cc. of ethyl ether, freshly distilled from sodium, were added 15 gm. of phosphorus pentachloride. The reaction mixture was shaken at room temperature for 20 minutes, filtered, and the filtrate concentrated *in vacuo*. The crystalline acid chloride was washed with petroleum ether, dissolved in 100 cc. of

anhydrous ethyl acetate, and this solution was added in two portions to a solution of DL-phenylalanine ethyl ester (prepared from 7.0 gm. of the hydrochloride) in 150 cc. of anhydrous ethyl acetate. Simultaneously with the addition of the second portion of the acid chloride, an aqueous solution of 5.0 gm. of  $\text{NaHCO}_3$  was added. The reaction mixture was shaken at room temperature for 30 minutes and then washed successively with 1 N HCl, saturated  $\text{NaHCO}_3$ , and water. After drying the ethyl acetate solution over anhydrous  $\text{Na}_2\text{SO}_4$ , the solution was concentrated *in vacuo*. The ester was saponified by dissolving it in 75 cc. of 1 N NaOH and allowing the solution to stand at room temperature for 1 hour. After acidification, the solution was concentrated *in vacuo* to yield a solid which was taken up in saturated  $\text{NaHCO}_3$ . Upon acidification, an oil separated which crystallized on standing. After recrystallization from ethanol and petroleum ether the yield was 7.0 gm.; m.p., 159–160°.

Calculated, N 7.73; found, N 7.70

*Formyl-L-phenylalanine* (FP) was prepared according to Fischer and Schoeller (19); m.p., found, 164°; reported (19), 165°.

Calculated, N 7.27; found, N 7.25

*Benzoylsarcosyl-DL-phenylalanine* (BSarP)—Sarcosyl-DL-phenylalanine was prepared by dissolving 5.0 gm. of chloroacetyl-DL-phenylalanine in 150 cc. of 25 per cent aqueous methylamine and allowing the reaction mixture to stand at room temperature for 4 days. The solution was concentrated *in vacuo*, the resulting solid extracted thoroughly with alcohol, and the product benzoylated in the usual manner. After recrystallization from an ethanol-water mixture the yield was 4.5 gm.; m.p., 202–203°.

Calculated, N 8.23; found, N 8.13

*Hippuryl-DL-phenylglycine* (HipPG)—This compound was prepared by adding benzoylglycine azide (14), prepared from 5.0 gm. of the hydrazide, to a slightly basic solution of 4.5 gm. of DL- $\alpha$ -aminophenylacetic acid in 200 cc. of water at 60–70°. After stirring for 5 minutes, the hot reaction mixture was filtered. After cooling, the filtrate was acidified and a gummy oil separated, which solidified on standing. After recrystallization from an ethanol-water mixture the yield was 2.5 gm.; m.p., 212°.

Calculated, N 8.96; found, N 8.89

*Hippuryl-L- $\beta$ -phenyllactic Acid* (HipPLA)—This compound was prepared in the same fashion as the racemate (11). Neutralization equivalent found, 332; calculated, 327. The racemate used in this investigation was the same as that previously described (11).

### Methods

**Peptidase Activity**—After equilibration of the substrate solution at 25° in 0.04 M phosphate buffer, pH 7.50, containing 0.1 M LiCl (7), the enzyme solution was added, care being taken to avoid contact of enzyme and phosphate prior to the experiment (20). At various time intervals, 0.2 cc. aliquots were withdrawn and pipetted into 2.0 cc. of the hot ninhydrin solution (21). The color was developed by heating in a water bath for 20 minutes, then diluted to 25 cc., and the optical density read in a Coleman junior spectrophotometer at 572 m $\mu$ .

Since the kinetic constants,  $K_m$  and  $k'$  (8, 12, 22), are functions of only the initial substrate concentration, enzyme concentration, and the initial velocity, the enzyme concentrations were chosen so that only about 5 per cent hydrolysis occurred within 2 hours. Up to 5 per cent hydrolysis, a linear relationship was obtained by about 10 experimental points when time was plotted against amount of substrate hydrolyzed. Substrate concentrations were determined by the semimicro-Kjeldahl method and enzyme concentrations by measurement of the optical density in a Beckman spectrophotometer at 278 m $\mu$ .

**Esterase Activity**—The preparation of the enzyme solutions as well as measurements of esterase activity were the same as those previously described (11, 23).

### Results

The kinetic constants,  $K_m$  and  $k'$ , were determined graphically as previously described (8, 12, 22). The results of measurement of the rate of hydrolysis of specific peptide substrates are given in Table I in which, for comparison, previous data on the hydrolysis of carbobenzoxyglycyl-DL-phenylalanine (CGP) are also included (8). As in the case of CGP, the hydrolysis of HipP is restricted to one optical isomer, presumably the L form, and the approximated first order reaction constants show a dependence on concentration.

Although the hydrolysis of chloroacetyl-L-phenylalanine is inhibited by chloroacetate (2, 5, 7), the degree of inhibition is negligible during the initial phase of the reaction, as evidenced by the linear relations of the velocity plots which were also obtained with acetyl- and formylphenylalanine.

The rate of hydrolysis of BSarP was too slow to be expressed in terms of  $K_m$  and  $k'$ . However, comparison of the velocity measured at a single initial substrate concentration of 0.04 M with that observed at a comparable substrate concentration of HipP shows that in the presence of 300 times as much enzyme N the reaction velocity is still only one-eighth that



TABLE I  
Kinetic Data for Hydrolysis of Specific Peptide and Ester Substrates by Carboxy-peptidase at 35°

Substrate	$a$	$v$	$e$	$K_m$	$k'$
	$10^{-3} M$	$10^{-6} M$	$10^{-3}$	$M$	
Carbobenzoxycylglycyl-DL-phenylalanine				0.033	2.1
Hippuryl-DL-phenylalanine	5.15	11.8	0.069	0.011	1.95
"	3.87	9.45			
"	2.58	9.2			
"	1.29	7.2			
Benzenesulfonylglycyl-DL-phenylalanine	4.26	7.85	0.835	0.014	0.124
"	3.20	7.4			
"	2.13	6.05			
"	1.07	5.0			
Formyl-L-phenylalanine	3.43	7.15	20.9	0.036	0.007
"	2.58	6.05			
"	1.72	4.6			
"	0.86	2.9			
Acetyl-DL-phenylalanine	3.53	2.7	54.3	0.155	0.0023
"	2.64	1.85			
"	1.76	1.28			
"	0.88	0.68			
Chloroacetyl-DL-phenylalanine	4.30	4.33	0.406	0.013	0.137
"	3.23	4.00			
"	2.16	3.55			
Benzoylsarcosyl-DL-phenylalanine	4	1.2	20.9		
Hippurylphenylglycine	4.0	4.4	1.04		
"	3.0	3.0			
"	2.0	1.8			
Hippuryl-L- $\beta$ -phenyllactic acid	0.25	43.0	0.250	$\sim 0$	1.72
Acetyl-DL- $\beta$ -phenyllactic acid	1.62	11.6	20.8	0.013	0.044
"	1.22	10.1			
"	0.81	8.0			
Chloroacetyl-DL- $\beta$ -phenyllactic acid	1.0-0.25	267	2.03	$\sim 0$	1.30
Bromoacetyl-DL- $\beta$ -phenyllactic acid	1.53	134	1.50	0.0016	0.99
"	0.718	122			
"	0.410	110			

$a$  = initial substrate concentration in moles per liter (with respect to the L isomer).  $v$  = initial velocity in moles per liter hydrolyzed per minute.  $e$  = enzyme concentration in mg. of enzyme N per cc.  $K_m$  = enzyme-substrate equilibrium constant; for definition see (8, 12).  $k'$  = specific rate of activation in moles per liter of substrate hydrolyzed per minute per mg. of enzyme N per cc.

of the latter. Hence, the specific rate of activation of BSarP is only about 1/2400 that of HipP.

Rate measurements of the hydrolysis of hippurylphenylglycine did not yield to simple kinetic interpretations. Negative values for  $K_m$  and  $k'$  were obtained when the data were plotted in the usual manner (8, 12, 22), and more extensive measurements are required to elucidate the reaction mechanism involving this substrate.<sup>2</sup> At an initial substrate concentration of 0.04 M, the reaction velocity is about 35 times slower than that of HipP at the same initial substrate concentration.

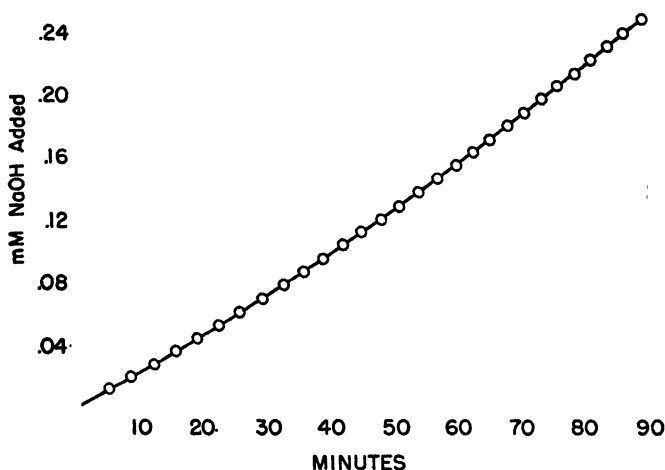


Fig. 1. Hydrolysis of hippuryl-*l*- $\beta$ -phenyllactic acid in 0.01 M phosphate buffer 0.025 M LiCl, pH 7.50, at 0°, by carboxypeptidase (0.057 mg. of enzyme N per 10 cc of reaction mixture). The initial substrate concentration was 0.040 M.

### Esters

It has been previously noted (11) that the hydrolysis of hippuryl-*dl*- $\beta$ -phenyllactic acid follows apparent zero order kinetics but that the rate constant decreases with increasing substrate concentration. Essentially the same results were obtained in the present investigation with the *l* isomer of HipPLA. In low substrate concentrations (0.025 M) and at 25°, the specific rate of activation is comparable to that of the corresponding peptide, *i.e.* HipP. At 0°, however, the hydrolysis of HipPLA does not follow the kinetics of a zero order reaction, as is shown by the plot given in Fig. 1.

<sup>2</sup> If the velocity data for HipPG, given in the third column of Table I, are approximated to the nearest whole number, the resulting values would be as expected for true first order reaction kinetics. This can only be obtained if the affinity of the enzyme for the substrate is so low that in the present range of substrate concentration the rate of enzyme-substrate combination becomes the rate-limiting step (8).

Representative data for the hydrolysis of chloroacetyl-*dl*- $\beta$ -phenyllactic acid (ClAcPLA) at four different initial substrate concentrations by a constant enzyme concentration of  $2.03 \times 10^{-3}$  mg. of enzyme N per cc. are shown in Fig. 2. Within the limits of the experimental error, the initial rate of hydrolysis approximates zero order kinetics. Maximum hydrolysis corresponds to one-half of the total concentration of the racemate.\* In order to determine whether the shape of the hydrolysis curves in Fig. 2 reflects the shift in equilibrium between the free and combined enzyme during the reaction (8), or is due to inhibition by chloroacetate (2,

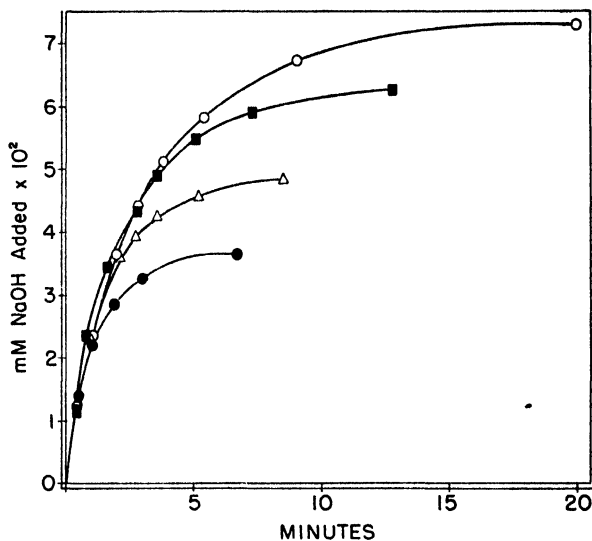


Fig. 2. Hydrolysis of chloroacetyl-*dl*- $\beta$ -phenyllactic acid by carboxypeptidase, at 25°. Buffer concentration as stated in legend to Fig. 1. Enzyme concentration, 0.0203 mg. of N per 10 cc. The initial substrate concentration (with respect to the *l* isomer) is shown by O, 0.008 M; ■, 0.0065 M, △, 0.005 M, ●, 0.004 M.

5), the hydrolysis of ClAcPLA in the presence of varying concentrations of chloroacetate was determined. The results are plotted in Fig. 3 and show that the rate of hydrolysis was independent of chloroacetate concentration, the different maximum levels of hydrolysis being due only to the variation in substrate concentration.

The enzymatic hydrolysis of bromoacetyl-*dl*- $\beta$ -phenyllactic acid was unusual in that the rate followed second order reaction kinetics. Representative data are given in Table II for measurements at three different

\* These results supersede preceding, preliminary observations (11) in which not more than 17 per cent hydrolysis of the racemate (i.e. 34 per cent of the susceptible isomeric form) was observed. The present results are based on more detailed and complete analyses.

enzyme concentrations. This order of reaction has no apparent relation to enzyme-substrate affinity, since the calculated value of  $K_m$  (Table I) is lower than that for the hydrolysis of HipP, which approximates first order reaction kinetics, whereas the specific rates of activation are of comparable magnitudes.

The results for all four specific ester substrates are included in Table I.

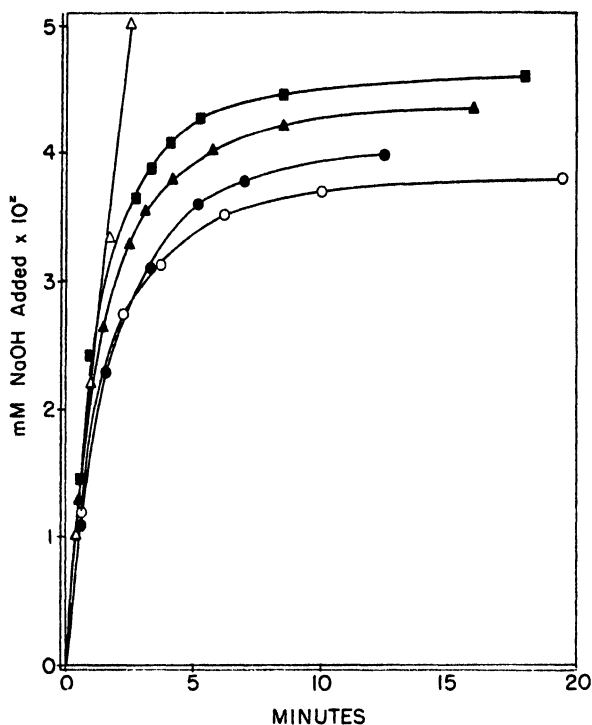


FIG. 3. Hydrolysis of chloroacetyl-*dl*- $\beta$ -phenyllactic acid by carboxypeptidase, at 25°, in the presence of chloroacetate. Enzyme concentration, 0.0172 mg. of N per 10 cc. The symbols refer to the following reaction mixtures:  $\Delta$  0.015 M substrate (*dl*);  $\blacksquare$  0.010 M substrate (*dl*) + 0.01 M chloroacetate;  $\blacktriangle$  0.009 M substrate (*dl*) + 0.005 M chloroacetate;  $\bullet$  0.009 M substrate (*dl*);  $\circ$  0.008 M substrate (*dl*) + 0.02 M chloroacetate.

### *Effect of Temperature*

The dependence of the rate of hydrolysis of ClAcPLA on temperature was measured in initial substrate concentrations of 0.02 and 0.01 M (concentrations of the racemate), respectively, at five different temperatures ranging from 0–30°. At all temperatures studied, the initial reaction rate was identical for both substrate concentrations. The results are plotted

TABLE II

Hydrolysis of Bromoacetyl-dl- $\beta$ -Phenyllactic Acid by Carboxypeptidase at pH 7.5, 25°

Time	$x$	$k$	Time	$x$	$k$	Time	$x$	$k$
$e = 2.0 \times 10^{-3}, a = 8.7 \times 10^{-3}$			$e = 1.5 \times 10^{-3}, a = 8.00 \times 10^{-3}$			$e = 1.0 \times 10^{-3}, a = 8.40 \times 10^{-3}$		
min.	$10^{-3}$		min.	$10^{-3}$		min.	$10^{-3}$	
1.6	2.63	29.2	1.5	1.80	24.2	1.4	1.34	16.1
2.4	3.29	29.1	2.3	2.42	23.6	2.2	1.88	15.6
3.8	4.29	29.5	3.5	3.09	22.5	3.2	2.42	15.1
5.4	5.00	28.8	4.6	3.65	22.8	4.5	3.04	15.0
7.3	6.60	27.6	6.0	4.20	23.0	6.1	3.69	15.3
10.4	6.27	28.6	8.1	4.77	22.8	8.1	4.27	15.2
13.9	6.73	28.2	10.6	5.28	22.9	10.8	4.86	15.6
19.1	7.20	28.9	14.1	5.78	23.0	14.5	5.46	15.3
32.0	7.70	27.7	19.8	6.29	23.2	19.3	5.98	15.2
			30.5	6.79	23.0	24.9	6.38	15.1
						30.5	6.63	14.6
Average		28.8			23.1			15.4
$\frac{k}{e}$	14,400			15,400			15,400	

$k = x/(ta(a-x))$ . The dimensions of  $k$  are liters  $\times$  mole $^{-1}$   $\times$  min. $^{-1}$ .  $a$  = initial substrate concentration in moles per liter (with respect to the  $l$  isomer).  $x$  = moles per liter of substrate hydrolyzed in time  $t$ .  $e$  = enzyme concentration in mg. of enzyme N per cc.

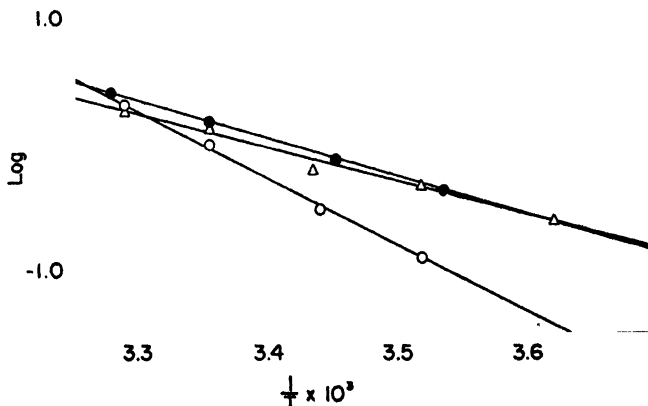


FIG. 4. A plot of  $\log k'$  against reciprocal temperatures (absolute) for the calculation of Arrhenius energies of activation for the hydrolysis of specific esters by carboxypeptidase. The symbols defining the straight lines refer to the following substrates:  $\circ$  0.015 M hippuryl-dl- $\beta$ -phenyllactic acid;  $\bullet$  0.005 M hippuryl-dl- $\beta$ -phenyllactic acid;  $\Delta$  0.02 and 0.01 M chloroacetyl-dl- $\beta$ -phenyllactic acid. All concentrations refer to the racemic mixtures. Experiments in 0.01 M phosphate buffer, 0.025 M LiCl; pH 7.50.

in Fig. 4. The calculated Arrhenius activation energy was 12,700 calories per mole.

Analogous measurements on HipPLA in initial substrate concentrations of 0.015 and 0.005 M (concentrations of the racemate), respectively, are likewise plotted in Fig. 4. The dependence of the specific rate of activation on temperature,  $k'$ , is greater for the higher initial substrate concentration, the calculated Arrhenius activation energies being 23,900 and 13,600 calories per mole, respectively.

#### DISCUSSION

The enzymatic hydrolysis of all but one (*i.e.* hippurylphenylglycine) of the peptide substrates listed in Table I follows the general reaction kinetics previously exemplified by the typical substrate carbobenzoxyglycylphenylalanine (8). The rates of reversible formation of the enzyme-substrate complex and the rates of activation of the complex are of comparable orders of magnitude, resulting in finite values of  $K_m$  and  $k'$ .

The enzymatic hydrolysis of the specific ester substrates, hippuryl- $l$ - $\beta$ -phenyllactic acid and chloroacetyl- $dl$ - $\beta$ -phenyllactic acid, follows apparent zero order kinetics, at least during the initial phase, indicating that in this range the sole rate-determining step is the activation of the complex (8, 12). The esterase activity of carboxypeptidase resembles, in this respect, that of trypsin (23).

The dependence of the hydrolysis of hippuryl- $l$ - $\beta$ -phenyllactic acid on concentration is similar to that previously described for the racemate and, hence, cannot be ascribed to antipodal inhibition. Since neither small amounts of the reaction products, *i.e.* hippuric acid and  $\beta$ -phenyllactic acid (11), the most likely impurities in the substrate preparation, nor variations in salt concentration (0.025 to 0.25 M LiCl) affect the rate of reaction, none of the factors previously considered (11) can account for the dependence of hydrolysis rate on substrate concentration. There is strong evidence for the view that HipPLA acts both as a substrate and an inhibitor for carboxypeptidase, a phenomenon not unfamiliar in the study of enzyme kinetics (24, 25). The observed zero order kinetics at 25° may be regarded as a resultant of two effects; *i.e.*, (1) the rate of hydrolysis proper, which decreases during hydrolysis because of the decreasing concentration of the enzyme-substrate complex, and (2) an accelerating effect due to decreasing inhibition as substrate concentration decreases. At 0°, the accelerating effect predominates, as is evidenced by an increase in the initial zero order reaction rate (Fig. 1), indicating that the combination of the enzyme with HipPLA as a substrate is more exothermic than the combination with the same compound as an inhibitor.<sup>4</sup>

<sup>4</sup> The assumption that the dependence of hydrolysis rates on concentration is due to adventitious impurities other than the reaction products is incompatible with these experimental findings.

The present data afford a more detailed analysis of the specificity of carboxypeptidase than has hitherto been possible on the basis of limited kinetic measurements.

The affinity of HipP for the enzyme is even greater than that of CGP, as is evidenced by a lower value of  $K_m$  in the face of practically identical rates of activation. Since the synthesis of HipP is simpler than that of the carbobenzoxyglycyl analogue and does not require the hazardous use of phosgene, it is to be recommended as the peptide substrate of choice.

While the nature of the aromatic radical adjacent to the *secondary* peptide bond appears to be rather inconsequential for activation rates, substitution of a sulfamide group for the secondary peptide bond, as in BSGP, causes a 16-fold decrease in the rate of activation. This is in contrast to analogous studies of trypsin which hydrolyzes  $\alpha$ -*p*-toluenesulfonyl-L-argininamide about 1.5 times faster than the corresponding  $\alpha$ -benzoyl analogue (23).

The hydrogen atom of the secondary peptide bond appears essential for rapid hydrolysis, since its replacement in HipP by a methyl group (BSarP) decreases the hydrolysis rate by a factor of 2400. This result is in essential agreement with analogous studies on substrates for chymotrypsin and may be explained by the same mechanism (22, 26). While AcP, which lacks entirely the secondary peptide bond, and is only slowly hydrolyzed ( $k'$  being 1/840 of that of HipP), the introduction of a chlorine atom, as in ClAcP, increases the activation rate 60-fold. A similar, though smaller, effect is evidenced in the corresponding ester substrates.

The relatively slow rate of activation of HipPG, compared to HipP (relative rates, approximately 1:35), is in full accord with studies on specific inhibitors (7), which have demonstrated the critical importance of the distance of separation of the phenyl ring from the carboxyl group.

The activity of carboxypeptidase toward ester analogues of specific substrates is convincingly demonstrated by the fact that, with one exception (*i.e.* HipPLA), the ester substrates are hydrolyzed faster than are the corresponding peptide substrates. In addition, the affinity of the enzyme for the esters appears to be higher than for the corresponding peptides, as evidenced by a comparison of the respective  $K_m$  and  $k'$  values (Table I) and by the preponderant adherence to zero order kinetics. This higher affinity is also evidenced by the failure of chloroacetate to inhibit the hydrolysis of ClAcPLA and HipPLA (11), in contrast to its inhibitory action on peptide hydrolysis (2, 5).

Comparison of the  $K_m$  and  $k'$  values for the present substrates provides a relative measure of their affinities (22), and leads to the following sequence of decreasing affinities for carboxypeptidase.



In each group of peptide or ester substrates, respectively, the affinity of the substrate for the enzyme decreases in the order hippuryl- > chloroacetyl- > (formyl-) > acetyl-. For substrates devoid of the secondary peptide bond, this sequence bears formal relation to the acidity of the corresponding acids which are formed on hydrolysis (27). However, substrates containing the secondary peptide bond are more firmly attached to the enzyme than are those devoid of this bond, despite the fact that corresponding acyl acids (carbobenzoxyglycine and hippuric acid) are weaker acids than chloro- or bromoacetic acids. The higher affinity of these compounds probably arises from the fact that they possess an additional point of attachment to the enzyme through the secondary peptide group by the dual action of this group in forming hydrogen bridges by acceptance and donation (22, 26).

A similar sequence exists when the peptide and ester substrates are arranged in order of decreasing specific rate of activation,  $k'$ . For substrates

TABLE III  
*Thermodynamic Data for Hydrolysis of Substrates by Carboxypeptidase\**

Substrate	$k'$	$k'_0$ <i>sec.<sup>-1</sup></i>	$\Delta E$ <i>calories per mole</i>	$\Delta H^*$ <i>calories per mole</i>	$\Delta S^*$ <i>E.U.</i>	$\Delta F^*$ <i>calories per mole</i>
ClAcPLA.....	1.30	111	12,700	12,100	-8.8	14,700
CGP.....	2.18	186	16,200	15,600	+3.6	14,500

\* Calculated for  $T = 25^\circ$ .

devoid of the secondary peptide bond, the sequence of specific activation rates of enzymatic hydrolysis parallels the sequence of the *alkaline* hydrolysis of the corresponding esters, while the influence of  $\alpha$  substituents on *acid* hydrolysis is considerably less and gives rise to an entirely different order than alkaline hydrolysis (28). Since, however, the specific activation rate of the typical substrate CGP by carboxypeptidase increases with increasing acidity of the system (between pH 7.8 and 6.5) (29), a correlation between the rates of enzymatic hydrolysis and the electronic properties of the acyl substituent of the substrate (27) is not self-evident. The higher specific rates of activation of substrates containing the secondary peptide bond as well as the slow rates of hydrolysis of BSGP and BSarP show that this structural element is necessary in order to obtain the full catalytic effect of the enzyme, and must again be related to the ability of this group to combine with the enzyme.

The measurements of the temperature dependence of the specific rate of activation,  $k'$ , plotted in Fig. 4, may be interpreted in terms of the energetic constants  $\Delta H^*$ ,  $\Delta S^*$ , and  $\Delta F^*$ , the changes in heat, entropy, and



free energy of activation, respectively (12). Such calculations appear valid for chloroacetyl-*dl*- $\beta$ -phenyllactic acid, since the specific rate of activation is independent of substrate concentration. Table III gives the results of such calculations, in comparison with analogous data for the hydrolysis of CGP (29).

Although the peptide substrate containing a secondary peptide bond (CGP) is split somewhat faster than the ester substrate devoid of this bond (ClAcPLA), the heat of activation is some 3500 calories per mole higher. Since the free energies of activation are practically identical, it follows that the entropy change accompanying the activation of CGP is not only 12.5 E.U. higher than that accompanying the activation of ClAcPLA, but entropy is actually gained during the activation process.

This work has been supported by the United States Public Health Service, National Institutes of Health.

#### SUMMARY

The enzymatic hydrolysis of a series of specific peptide and ester substrates by carboxypeptidase has been investigated by quantitative kinetic measurements. Both the affinity for the enzyme and the rate of activation are higher for esters than for the corresponding peptides.

While the secondary peptide bond of specific substrates enhances enzymatic hydrolysis, compounds devoid of this structural component are likewise hydrolyzed, although at a slower rate. Their affinity for the enzyme is in direct relation to the electronic nature of the substituent.

*N*-Methylation of the secondary peptide bond causes a large decrease in the rate of hydrolysis; replacement of the secondary peptide group by a sulfamide group decreases hydrolysis rates to a lesser but significant extent.

The unusual reaction kinetics previously noted for the hydrolysis of hippuryl-*dl*- $\beta$ -phenyllactic acid have been confirmed with the *l* isomer of this compound and related to its dual action as both substrate and inhibitor for carboxypeptidase.

The influence of temperature on the specific rate of activation of chloroacetyl-*dl*- $\beta$ -phenyllactic acid has been determined and described in terms of energetic constants.

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## THE CHEMISTRY OF MELANIN

### VI. MECHANISM OF THE OXIDATION OF CATECHOL BY TYROSINASE\*

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The accompanying diagram illustrates a mechanism proposed for the enzymic oxidation of catechol (1-5). In the present study the oxidations of catechol and hydroxyhydroquinone at varying concentrations of substrate, enzyme, and hydrogen ion have been followed spectrophotometrically. Step 1, first proposed by Raper on chemical grounds (6), has been confirmed. Further results indicate that the catechol melanin obtained under these conditions is not a polymer of hydroxy-*p*-quinone.

#### EXPERIMENTAL

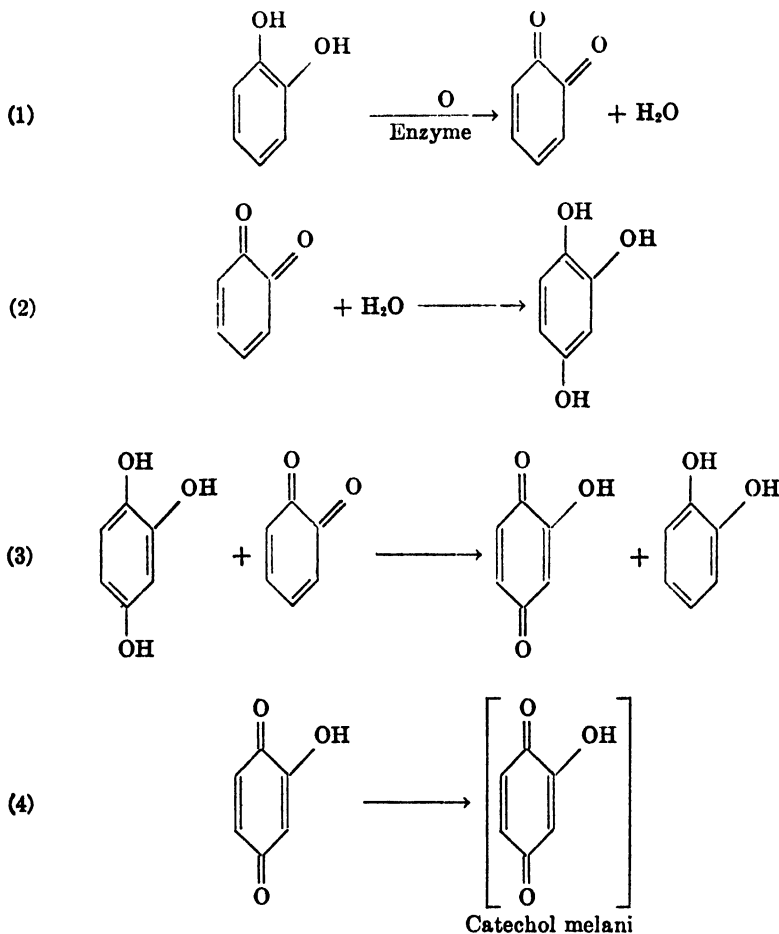
Tyrosinase was prepared from *Psalliotia campestris* by the procedure of Ludwig and Nelson (7). The preparations contained between 83 and 107 chrometric catecholase units per mg. of dry weight, determined by the procedure of Miller *et al.* (8). Catechol melted at 103-104° (corrected). *o*-Benzoquinone (9, 10) consisted of bright red needles which melted and decomposed between 60-70°. Hydroxyhydroquinone (11) melted at 139-140°. Hydroxy-*p*-quinone prepared from it (12) crystallized from ether in yellow prisms which melted at 122-125°. 2,4,5,2',4',5'-Hexahydroxydiphenyl was prepared from vanillin according to the procedure of Erdtman (13); the granular gray powder possessed the properties previously described (13-15). It was further identified by preparation of the corresponding hexaacetate, which melted at 172.5-174.5°. A dilute ethereal solution shaken with silver oxide and dry sodium sulfate first turned deep red-purple, then lemon yellow. Yellow crystals melting and decomposing at 180° were deposited from the concentrated filtrate. The small amounts available forestalled further identification, but the method of preparation and the color sequence observed during the reaction (*cf.* 4,4'-dimethoxydi-*p*-quinone of Erdtman) indicate that this substance was 4,4'-dihydroxydi-*p*-quinone.

Buffers were prepared by adding 0.1 N NaOH to 50 ml. of 0.1 M KH<sub>2</sub>PO<sub>4</sub>.

\* Some of the results reported in this paper were the subject of a preliminary note (*J. Am. Chem. Soc.*, **67**, 1233 (1945)). For Paper V in this series see Mason and Wright (*J. Biol. Chem.*, **180**, 235 (1949)).

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and diluting the mixture to 100 ml. All spectrophotometric experiments were conducted at temperatures of 25–28° with a Beckman ultraviolet spectrophotometer and matched quartz cells having a light path of 1.0 cm. The constant procedure of adding 0.1 ml. of enzyme, appropriately diluted, to 3.0 ml. of substrate-buffer mixture in the spectrophotometer



cell was utilized. The spectrophotometric conventions and experimental details have been previously described (16, 17).

**Enzymic Oxidation of Catechol**—The spectrophotometric course of the oxidation of catechol in the presence of tyrosinase at pH 5.4 is depicted in Fig. 1. Although the concentration of catechol is varied between  $7.54 \times 10^{-5}$  and  $2.27 \times 10^{-4}$  mole per ml. and the concentration of tyrosinase

between 5.3 and 21.2 catecholase units per 3.1 ml., the observations are qualitatively alike. The absorption maximum at 276  $m\mu$ , characteristic

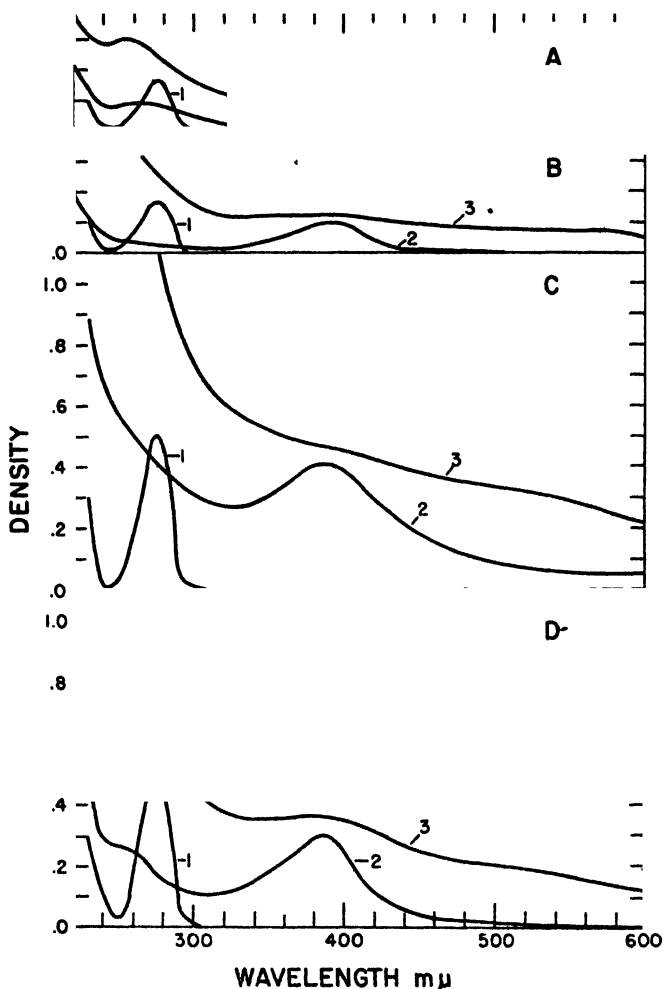


FIG. 1. Spectrophotometric course of the enzymic oxidation of catechol at pH 5.4. In *A* and *B*,  $7.54 \times 10^{-4}$  mole per ml. and, in *C* and *D*,  $2.27 \times 10^{-4}$  mole per ml. of catechol were oxidized in the presence of 21.2 (*A* and *C*) and 5.3 catecholase units of enzyme (*B* and *D*). In each section Curve 1 represents the absorption spectrum observed before addition of enzyme, Curve 2, immediately after addition of enzyme, and Curve 3, 60 minutes after.

of catechol, is replaced rapidly by principal absorption at 390  $m\mu$ , which will presently be shown to be characteristic of *o*-benzoquinone. General

absorption displaying a weak maximum at 255 to 260  $m\mu$  then develops over a period of 60 to 90 minutes. The results obtained with the same range of enzyme and substrate concentrations at pH 8.4 are depicted in

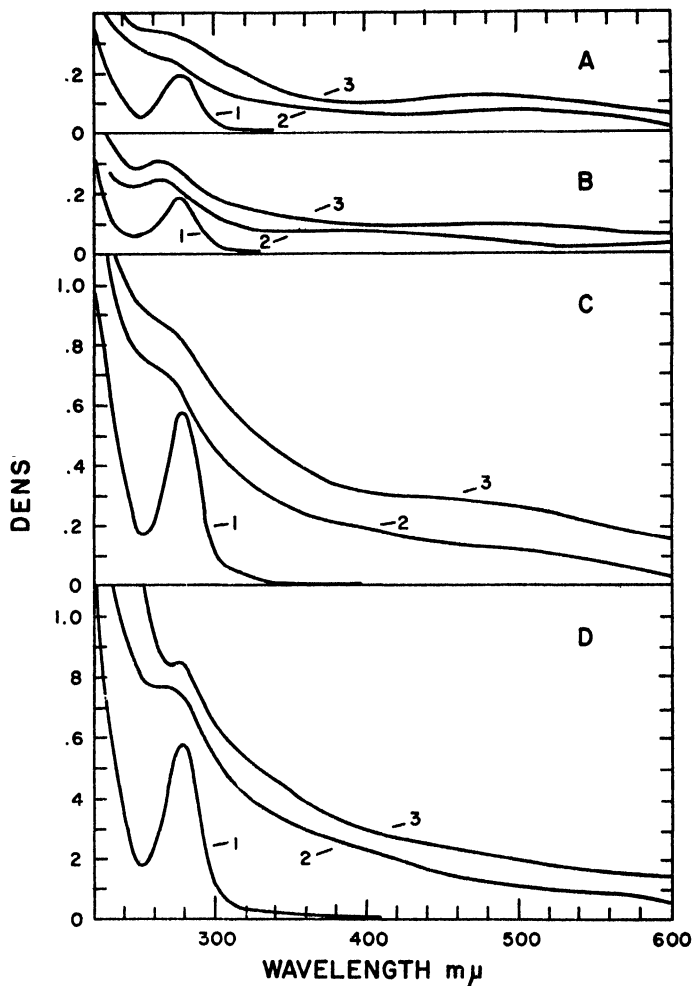


FIG. 2. Spectrophotometric course of the enzymic oxidation of catechol at pH 8.4. The designations have the same significance as those in Fig. 1.

Fig. 2. Intermediate spectra are more transient; the maximum absorption of catechol at this pH, 277.5  $m\mu$ , is directly replaced by general absorption displaying weak maxima or inflections at 260 to 270  $m\mu$ .

*Enzymic Oxidation of Hydroxyhydroquinone*—The spectrophotometric course of the oxidation of hydroxyhydroquinone in the presence of tyrosinase

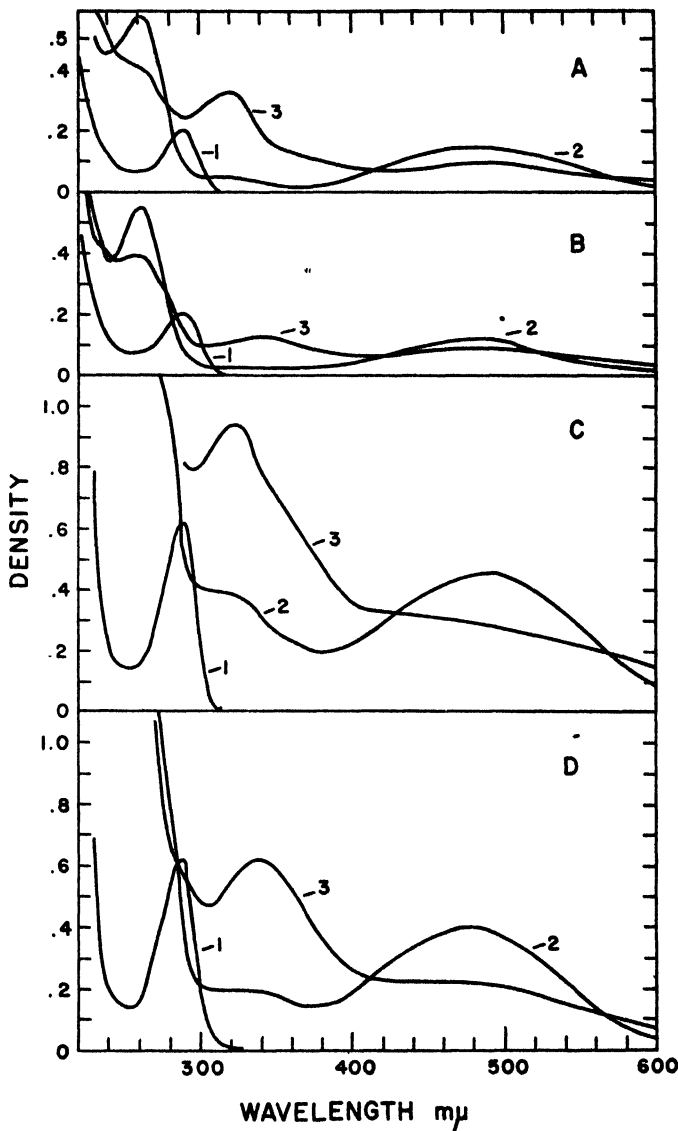


FIG. 3. Spectrophotometric course of the enzymic oxidation of hydroxyhydroquinone at pH 5.4. The designations have the same significance as those in Fig. 1.

at pH 5.4 is depicted in Fig. 3. The concentrations of enzyme and substrate are the same as in the previous experiments with catechol. In each, the initially observed maximum at 288 mμ, characteristic of hydroxyhydroquinone, is rapidly replaced by two new maxima at 260 and 480 to

485  $m\mu$ . These will presently be shown to constitute a portion of the absorption spectrum of hydroxy-*p*-quinone. Under the conditions of the experiment they are metastable and are replaced in 60 minutes by a band, the position of which is related to the amount of enzyme in solution: 320

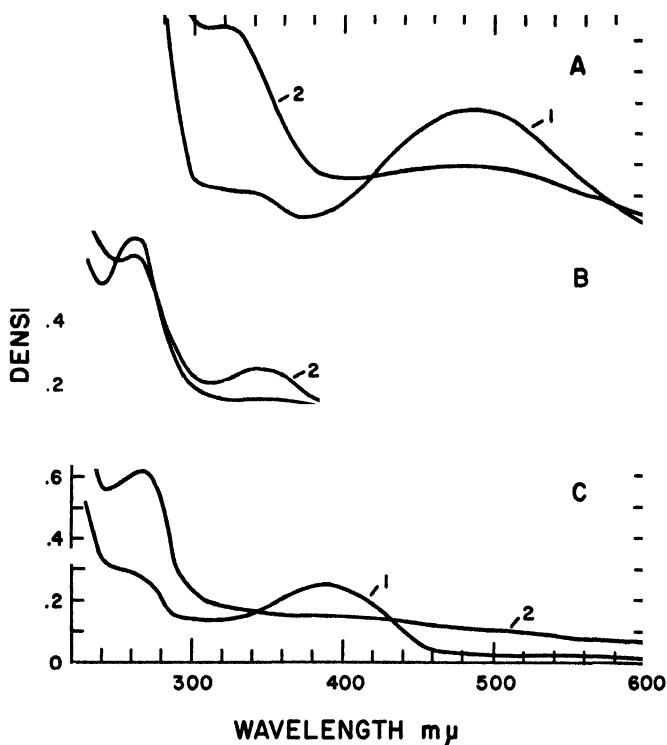


FIG. 4. Serial changes in the absorption spectra of hydroxyhydroquinone, hydroxy-*p*-quinone, and *o*-benzoquinone. *A*, hydroxyhydroquinone,  $2.27 \times 10^{-4}$  mole per ml., at pH 8.4 observed (Curve 1) immediately, and (Curve 2) 60 minutes after solution of the compound. *B*, hydroxy-*p*-quinone,  $1.41 \times 10^{-4}$  mole per ml., at pH 5.4 observed (Curve 1) immediately, and (Curve 2) 60 minutes after solution of the compound. *C*, *o*-benzoquinone,  $2.05 \times 10^{-4}$  mole per ml., at pH 5.4 observed (Curve 1) immediately, and (Curve 2) 60 minutes after solution of the compound.

$m\mu$  in the presence of 21.2 units and 340  $m\mu$  in the presence of 5.3 units. Increases in the concentrations of hydroxyhydroquinone and enzyme produce expected increases in the rate of appearance and intensities of the several bands serially observed.

At pH 8.4 the rate of autoxidative transformation of hydroxyhydroquinone is too rapid to be observably affected by the presence of enzyme



(Fig. 4, A). Immediately after solution of the phenol, absorption maxima at 260 and 480  $m\mu$  are observed. These are replaced more slowly by a single maximum at 320  $m\mu$ .

*Enzymic Oxidation of Mixtures of Catechol and Hydroxyhydroquinone*—Mixtures of catechol and hydroxyhydroquinone, the combined concentrations of which equaled  $2.27 \times 10^{-4}$  mole per ml., were oxidized in the presence of 21.2 units of enzyme at pH 5.4. The absorption spectra which developed immediately after addition of the oxidase contained bands at both 395 and 490  $m\mu$ , the relative intensities of which were proportional to the mole fractions of catechol and hydroxyhydroquinone, respectively. General absorption then appeared. When the concentration of hydroxyhydroquinone was 50 per cent or more of the total sub-

TABLE I  
*Absorption Maxima and Molecular Extinction Coefficients of Some Phenols and Quinones*

Compound	pH of buffer solvent	Maxi- mum  $m\mu$	Log <i>E</i>	Maximum  $m\mu$	Log <i>E</i>
Catechol.....	5.4	276	3.362		
" .....	8.4	277.5	3.410		
<i>o</i> -Benzoquinone.....	5.4	390	3.262		
" (enzymic).....	5.4	390	3.124		
Hydroxyhydroquinone.....	5.4	288	3.441		
Hydroxy- <i>p</i> -quinone.....	5.4	260	3.669	480-485	3.312
" (enzymic).....	5.4	260	3.690	480-485	3.324
2,4,5,2',4',5'-Hexahydroxydiphenyl..	5.4	292	4.019		
4,4'-Dihydroxydi- <i>p</i> -quinone.....	5.4	275	4.260		

strate concentration, an inflection or maximum simultaneously appeared in the region of 340  $m\mu$ .

*Absorption Spectra of Hydroxy-*p*-quinone and *o*-Benzoquinone*—The absorption spectrum of hydroxy-*p*-quinone at pH 5.4 is depicted in Fig. 4, B. Unstable maxima are observed at 260 and 480 to 485  $m\mu$ ; over a period of 60 minutes these diminish in intensity and a new band at 340  $m\mu$  appears. *o*-Benzoquinone displays an initial maximum at 390  $m\mu$ ; this rapidly disappears and general absorption in which a well defined maximum at 270  $m\mu$  is apparent develops (Fig. 4, C). The absorption maxima and molecular extinction coefficients of catechol, hydroxyhydroquinone, the corresponding quinones, and related substances relevant to the following discussion and observed in the present study are listed in Table I.

## DISCUSSION

The isolation of 4,5-dianilino-*o*-benzoquinone as a derivative from the products of the oxidation of catechol in the presence of tyrosinase (2, 6) indicated that *o*-benzoquinone is formed under these conditions. The present study provides confirmation, since the absorption spectrum observed immediately after addition of tyrosinase to catechol solutions and that of *o*-benzoquinone itself are identical at pH 5.4 (Table I). *o*-Benzoquinone must therefore be formed more rapidly than it is consumed in subsequent reactions at this pH. At pH 8.4, however, the quinone must be utilized as rapidly as it forms, for only general absorption can be observed in the region of 390 m $\mu$  after addition of enzyme to catechol solutions (Fig. 2).

The final stages of the enzymic oxidation of catechol are characterized by inflections or maxima in the region of 255 to 270 m $\mu$ . These are generally more pronounced in the presence of low initial enzyme concentrations. Solutions of pure *o*-benzoquinone develop similar maxima upon standing (Fig. 4, C) and catechol itself can readily be isolated from such solutions. For this reason it is probable that these peaks do not characterize the polymeric product.

The enzymic oxidation of hydroxyhydroquinone follows a completely different spectrophotometric course. In the first phase the spectrum of this polyphenol is replaced by absorption maxima at 260 and 480 to 485 m $\mu$  (pH 5.4) (Fig. 3). Similar changes take place autoxidatively at pH 8.4. This spectrum is identical to that of hydroxy-*p*-quinone (Fig. 4, B; Table I).

The instability of this compound in aqueous solution does not, as in the case of *o*-benzoquinone, lead directly to general absorption but to new specific absorption in the region of 320 to 340 m $\mu$  (Figs. 3, 4, A, and 4, B). The appearance of this absorption band 60 minutes after the solution or enzymic formation of hydroxy-*p*-quinone is an additional characteristic of the compound at pH 5.4. Furthermore, the 320 to 340 m $\mu$  band develops perceptibly when the concentration of hydroxy-*p*-quinone is 50 per cent or more of the total in a mixture with *o*-benzoquinone formed by the enzymic oxidation of the corresponding phenols at pH 5.4. Since neither this band nor those characterizing hydroxy-*p*-quinone itself appear any time during the enzymic oxidation of catechol at pH 5.4 to 8.4, it is probable that the mechanism postulated by Nelson and coworkers does not obtain under these conditions.

The hypothesis that hydroxy-*p*-quinone is formed during the enzymic oxidation of catechol developed from the observation that, after the enzymic consumption of 2 atoms of oxygen per molecule of catechol, addition of aniline results in the formation of 4,5-dianilino-*o*-quinone (2).

However, it is known that hydroxy-*p*-quinone does not give rise to 4,5-dianilino-*o*-quinone (18). Accordingly, this basis for the hypothesis is without foundation. An alternative explanation for the isolation of 4,5-dianilino-*o*-quinone by Wagreich and Nelson lies in the observation that the complete enzymic oxidation of catechol requires 2.5 to 3.1 atoms of oxygen per molecule of catechol (19). The consumption of only 2.0 atoms per molecule may permit unchanged *o*-benzoquinone to remain. This would form the derivative obtained.

The kinetics of the disappearance of *o*-benzoquinone from dilute aqueous solutions has also been interpreted as indicative of a simple hydration reaction between the quinone and water (3). The analytical procedure employed for following the concentration of *o*-benzoquinone in this work did not, however, discriminate between *o*-benzoquinone initially present and quinones subsequently formed.

Under the conditions of the experiment, hydroxy-*p*-quinone did not condense to polyhydroxydiphenyls of the type described by Erdtman (13), since these substances do not possess absorption maxima in the 320 to 340  $m\mu$  region (Table I). While the products of the enzymic oxidation of hydroxyhydroquinone are readily dialyzable and do not precipitate upon the addition of strong acid, catechol melanins prepared under the same conditions are retained by a cellulose membrane and are almost completely precipitated from solution at pH 2. This evidence together with failure to detect intermediate substances spectroscopically suggests that *o*-benzoquinone polymerizes directly to phenolic polyphenyls susceptible to further oxidation.

#### SUMMARY

Comparison of the spectrophotometric course of the enzymic oxidations of catechol and hydroxyhydroquinone under varying conditions of substrate, enzyme, and hydrogen ion concentrations indicates that the catechol melanins obtained under these conditions are not polymers of hydroxy-*p*-quinone.

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# THE EFFECT OF PREVIOUS DIET ON THE METABOLIC ACTIVITY OF THE ISOLATED RAT DIAPHRAGM\*

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While there has been considerable evidence of a shift in the enzyme systems of unicellular organisms due to a continuing modification of the environment, no clear cut evidence of such a shift in mammalian tissues has been obtained. Indirect evidence of such a shift has been reported by Samuels, Roberts, and coworkers (1-5) in fasted and in eviscerated rats. Since the largest mass of tissue in the eviscerated animal is striated muscle, the metabolism *in vitro* of diaphragmatic muscle from rats previously fed equicaloric high carbohydrate and high fat diets was investigated. It was considered that any difference in metabolism under a constant environment would indicate a change in the metabolic mechanisms within the muscle cells.

## Methods

Male rats obtained from Sprague-Dawley, Inc., were used in all experiments. The animals were fed Purina fox chow checkers until they attained a weight of 225 gm. or more. In each experiment the rats were then segregated into two groups having similar weight distribution and fasted 24 hours. Forced feeding of either a high fat or high carbohydrate diet by the technique of Reinecke *et al.* (6) was then begun. Twice daily, at 8.00 a.m. and at 3.00 p.m., the rats were fed for a period of at least 4 weeks. The composition of the diets is given in Table I. The animals were fed sufficient diet to produce a gain in weight. The initial feeding was 8 ml. This was increased 1 ml. every other day until a total of 13 ml. at each feeding was given. This was further increased if necessary to maintain weight. Water was available *ad libitum*. The technique of preparing the diaphragms was similar to that of Gemmill (7). For use in the experiments,

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the rats were sacrificed by decapitation and bled. Immediately following the cessation of bleeding, the abdomen was opened and the diaphragm was removed and immersed in ice-cold 0.01 M Krebs-Ringer-phosphate buffer, pH 7.4, prepared as described by Umbreit (8). During this operation, stretching and handling of the diaphragm were kept at a minimum. All possible fat and connective tissue were removed and the diaphragm cut into pieces weighing approximately 50 mg.

Each piece of diaphragm tissue was blotted separately, quickly weighed on a torsion balance, and transferred to a Warburg vessel containing ice-cold 0.01 M Krebs-Ringer-phosphate buffer. Generally two pieces were

TABLE I  
*Diets Used in Stomach Tube Feeding*

	High fat	High carbohydrate
Lactalbumin, Labco, gm.....	30	30
Gelatin, U. S. P., gm.....	30	30
Corn oil, gm.....	105.8	3
Dextrin, gm.....	0	233.25
Salt Mixture 2, U. S. P., gm.....	12	12
Oleum percomorphum, reinforced, Mead Johnson, drops..	12	12
Vitamin concentrate, Lederle's Lederplex, gm.....	4	4
Water to make, ml.....	700	700
Calories per ml.....	1.68	1.70
Caloric distribution, % of total calories		
Protein.....	19.5	19.5
Fat.....	80	2.7
Carbohydrate.....	0.5	77.8

used in each flask. When desired, substrate was added to the buffer. The vessels containing buffer and tissue were next attached to Warburg manometers, equilibrated with the gas phase, placed in the bath at 37°, and equilibrated for 15 minutes. The manometers were then closed and the contents of the flasks shaken for a period of 2 hours.

A final concentration of 100 mg. per cent glucose was used for carbohydrate substrate in the determination of anaerobic lactate production. The vessels containing the tissue and reaction medium were gassed with 100 per cent nitrogen passed through a heated glass tube containing copper turnings; so that the last traces of oxygen were removed. After the incubation period, the vessels were removed from the manometers and a 1 ml. aliquot of the reaction medium was taken for determination of lactic acid by the method of Barker and Summerson (9).

For glucose utilization and glycogen synthesis, final concentrations of

133 and 200 mg. per cent glucose were used as substrate. The vessels were gassed with 100 per cent oxygen and equilibrated, and respiration continued for 2 hours after closing the manometers. After this period of respiration, the vessels were removed from the Warburg manometers and the tissues were lifted out, quickly rinsed in distilled water, and dropped into 30 per cent potassium hydroxide solution for determination of glycogen by the method of Good *et al.* (10). A 1 ml. aliquot of the buffer solution was taken for the determination of glucose by Reinecke's modification of the Folin-Malmros technique (11). Another 1 ml. aliquot was taken for determination of lactic acid, as previously described.

For initial glucose concentration, duplicate 1 ml. aliquots of the medium used were subjected simultaneously to the same procedure as were the unknowns. For control glycogen, approximately 100 mg. of tissue were

TABLE II

*Carbohydrate Utilization by Strips of Diaphragm from Rats on High Carbohydrate or High Fat Diets*

Results in mg. per gm. of tissue per hour.

Diet	Substrate	No. of animals	Change in glucose	Change in glycogen (as glucose)	Glucose balance
Carbohydrate...	Glucose + buffer	11	$-1.47 \pm 0.012$	$-0.30 \pm 0.004$	-1.77
Fat.....	" "	11	$-0.64 \pm 0.011^*$	$+0.38 \pm 0.008^*$	-0.26
Carbohydrate...	Buffer	9		$-0.64 \pm 0.003$	-0.64
Fat.....	"	9		$-0.34 \pm 0.004$	-0.34

\* Standard deviation of the mean.

placed in hot potassium hydroxide solution at the same time that similar pieces of the same diaphragm tissue were put into the Warburg vessels. This sample was then analyzed in the same manner as the incubated tissue.

### Results

The results of the study of glucose utilization are listed in Table II. When glucose was present in the buffer, the utilization of this compound was significantly higher in the diaphragms of the carbohydrate-fed rats than in those of the fat-fed animals. The mean initial glycogen content of the diaphragms was significantly higher in the carbohydrate-fed group. Glycogen was increased in most of the diaphragms of the fat-fed animals when glucose was present, while it was decreased under all conditions in those from the carbohydrate-fed animals. Furthermore, without added substrate the utilization of glycogen by the diaphragms of the fat-fed animals was significantly less than the glycogen utilized by diaphragm tissue of rats

fed the high carbohydrate diet. Thus, either in the presence of glucose at levels of 133 and 200 mg. per cent, or in its absence, there was always a greater disappearance of glucose and glycogen in the muscle tissue of the carbohydrate-fed group.

TABLE III

*Relation of Oxygen Consumption to Carbohydrate Utilization in Rat Diaphragms*  
Results in ml. per gm. per hour.

Diet	Substrate	No. of animals	O <sub>2</sub> consumption	Glucose* balance	O <sub>2</sub> equivalent of glucose balance	Non-glucose O <sub>2</sub> consumption
Fat.....	Glucose + buffer	11	1.04 ± 0.03†	-0.26	-0.19	+0.85
" .....	Buffer	9	0.93 ± 0.06	-0.34	-0.25	+0.68
Carbohydrate....	Glucose + buffer	11	0.88 ± 0.03	-1.77	-1.32	-0.44
" .....	Buffer	9	0.90 ± 0.05	-0.64	-0.48	+0.42

\* Change in glucose and glycogen.

† Standard deviation of the mean.

TABLE IV

*Lactic Acid Content of Medium after Aerobic and Anaerobic Incubation of Diaphragm*  
Results in mg. per gm. per hour.

Diet	Substrate	Anaerobic		Aerobic	
		No. of rats	Lactate	No. of rats	Lactate
Fat.....	Glucose + buffer	4	0.99 ± 0.11*	6	0.73 ± 0.03*
Carbohydrate....	" "	4	1.02 ± 0.03	6	0.63 ± 0.04
Fat. ....	Buffer only	4	0.83 ± 0.07	4	0.68 ± 0.02
Carbohydrate....	" "	4	0.86 ± 0.04	4	0.52 ± 0.04

\* Standard deviation of the mean.

As shown in Table III, there was no significant difference in oxygen uptake in the diaphragm of animals on the same diet when buffer alone or when glucose and buffer were used as substrate. Apparently the muscle tissue from rats on either high fat or high carbohydrate diet contains sufficient substrate to maintain oxygen consumption at a level which is not significantly affected by glucose in the surrounding medium.

In Table III the oxygen required to oxidize the carbohydrate which disappeared during incubation is compared with the total oxygen consumed. It must be concluded that some substrate other than glucose from the



medium or glycogen was oxidized by the diaphragms of the fat-fed rats even in the presence of glucose.

Table IV gives the concentration of lactic acid per gm. of muscle tissue. The presence of glucose increased the formation of lactic acid under both aerobic and anerobic conditions. Diet had no effect on the level of lactic acid found after anaerobic incubation. When the diaphragms were incubated aerobically, however, there was significantly more lactic acid in the muscles of the fat-fed rats.

#### DISCUSSION

The data from these experiments indicate that the oxidative metabolism of striated muscle from rats on a high fat diet differed from that observed after a high carbohydrate diet; the glycolytic system was apparently not significantly affected.

The difference in glycogen formation is in contrast with the results reported by Lundbaek and Stevenson (12). They found that glucose utilization by the tissues of the fat-fed rats was little more than half that of those from the carbohydrate-fed group, but no significant difference in glycogen synthesis was observed when the medium contained glucose at a level of 200 mg. per cent. The glucose utilization by the muscles of our fat-fed rats was similar, 44 per cent of that in the carbohydrate-fed animals, but we also observed differences in glycogen formation which contributed to the difference in total carbohydrate utilization. This may have been due to the fact that the initial level of glycogen was higher in the carbohydrate-fed rats. The age of the animals may also have been a factor. The difference in muscle glycogen was itself a reflection of the different substrate effect on the cells, since the total amount of protein and energy in the diet was the same in both groups.

Since anaerobic glycolysis by the diaphragms from these two groups of rats was similar, as was also oxygen uptake, while glucose utilization was much greater in the tissues from the carbohydrate-fed animals, there must be some other factor in muscle which can be utilized for energy. This statement is in agreement with the conclusions of Gemmill (7) based on R.Q. measurements and oxygen consumption of diaphragm tissue in the absence of glucose and insulin. This component is apparently utilized in greater amount in the diaphragm of the fat-fed rat. Gemmill's figures for oxygen consumption (0.84 to 1.06 ml. per gm. per hour is the range of means) also coincide with our observations.

Although the diaphragms from rats fed these diets may be able to utilize preferentially the products of metabolism from the major type of food-stuff which has been fed, apparently the ability of the tissue to utilize either type has not been abolished. This is demonstrated by the ability of the

tissues of the fat-fed rats to use small amounts of glucose when it was present and to utilize some carbohydrate as glycogen when no outside source of energy was provided.

The constant consumption of oxygen by diaphragm tissue from rats on the diet either with or without substrate indicates a relatively abundant supply of usable sources of energy within the striated muscle. This independence of substrate confirms the findings of Gemmill (13) and Gemmill and Hamman (14), among others. Since the diaphragm from the fat-fed rat, which has been demonstrated by the present work to use some substrate other than glucose or glycogen, is equally independent of its immediate nutritive surroundings, it seems that such a tissue also has considerable stores of an unknown oxidizable substance. An investigation of the distinctive constituents of the striated muscle of the fat-fed rat maintained as outlined here should lead to its identification. A study of the blood sugar curves of the eviscerated rat as published by Roberts, Samuels, and Reinecke (4) would indicate that this substrate is probably exhausted after several hours, since the glucose in the blood then begins to fall more rapidly.

According to Krah1 and Cori (15) the difference in utilization of glucose by the two groups of muscles in the presence of the same glucose level would be a measure of the activity of the hexokinase system. This is based on the virtual irreversibility of the system due to the great energy drop when a high energy phosphate bond is broken to form a hexose phosphate. Since insulin increases the activity of this system in muscle slices of normal rats, the assumption would then be that the differences observed in the present studies are due to differences in insulin content of the diaphragm. This may well be, since Best *et al.* (16) have shown that the insulin content of the pancreas is low on a high fat diet. There are, however, several factors which seem to make this questionable.

First is the assumption by Krah1 and Cori that the availability of high energy phosphate was not the limiting factor in the hexokinase reaction. No source of this was added in their experiments nor in ours. The original concentration and the replacement of adenosine triphosphate and adenosine diphosphate was, therefore, dependent on the over-all activity of the tissues themselves. It is doubtful whether this assumption should be made in any unknown system. Against any major difference in the hexokinase system is the constancy of the anaerobic glycolysis between the groups. This also involves glucose-6-phosphate formation and should be affected by a difference in hexokinase activity.

Certainly it appears that the major difference is in the oxidative cycle rather than in hexokinase. Insulin could, of course, be responsible for this.

The step at which the oxidative cycle differs and the effect of insulin are being studied.

Whether the differences measured here are those which seem to persist during subsequent fasting in the intact rat (17) remains to be tested by further experiments on diaphragms from such animals. The present differences may be due to variations in substrate concentration within the cell rather than in enzyme activity, since the diffusibility of the non-glucose substrate is unknown. These problems are now under investigation.

#### SUMMARY

The metabolism of diaphragm tissues of rats previously given a high fat or high carbohydrate diet by forced feeding for a period of at least 4 weeks has been compared *in vitro* by means of the following: (a) oxygen uptake, with either buffer alone or buffer plus glucose as substrate; (b) aerobic and anaerobic lactic acid production, with and without glucose present as substrate; (c) glucose utilization; and (d) glycogen synthesis, with and without glucose substrate.

Experimental evidence is presented showing that the diaphragm tissue of the rat which had been forcibly fed a high fat diet utilizes much less carbohydrate compared with that from a rat forcibly fed a high carbohydrate diet, while slightly more oxygen is consumed. Some substance other than glucose or glycogen readily available for energy must, therefore, be stored in the striated muscle of the rat forcibly fed a high fat diet.

When tissues were incubated for 2 hours, oxygen uptake on either diet was not significantly affected by adding 200 mg. per cent glucose to the medium; in the muscle of fat-fed rats, therefore, the non-glucose substance must be stored in adequate amounts to maintain normal oxidation for this length of time.

Anaerobic lactic acid production was not affected by these differences in the previous diet of the animal. After aerobic incubation lactic acid was significantly higher in the media containing diaphragms from the fat-fed rats.

The hexokinase and glycolytic systems do not appear to be a major factor in this alteration in metabolic activity. The difference appears to be in the oxidative phase of the cycle.

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# SODIUM AND ITS RELATION TO ALLOXAN DIABETES AND GLUTATHIONE\*

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An investigation into the effect of sodium on alloxan diabetes was prompted by the following observations. The beneficial effect of sodium chloride for diabetic persons was demonstrated by McQuarrie *et al.* (1). They found that an increased intake of sodium chloride resulted in a decrease in the fasting level of blood sugar and in glycosuria. Sayers *et al.* (2) showed that the low glucose tolerance of the Yale strain of rats was improved by a simultaneous injection of sodium chloride. Lewis *et al.* (3) confirmed these results when rats fed a high sodium chloride ration were shown to possess a greater glucose tolerance. In both cases the liver glycogen was found to be increased when sodium chloride was supplied.

Since sodium chloride improved the state of the diabetic, the possibility that the severity of alloxan diabetes would be increased by a lack of sodium became apparent. A preliminary experiment, in which sodium-deficient rats were injected with alloxan, indicated that sodium had a pronounced effect on the induction of this type of diabetes. Therefore, a more detailed study was made on the effect of sodium and potassium upon the induction of alloxan diabetes. The effect of potassium was studied, since it had been reported antagonistic to sodium (1, 3).

## EXPERIMENTAL

The basal ration, which contained 0.005 per cent sodium and 0.005 per cent potassium by flame photometric analysis, was based on that used by Sporn, Ruegamer, and Elvehjem (4). It was composed of sucrose 74.5 per cent, casein (Labco) 18 per cent, corn oil (Mazola) 5 per cent, Salts IV (sodium- and potassium-low) 2.5 per cent, and thiamine hydrochloride 0.3 mg., riboflavin 0.3 mg., pyridoxine hydrochloride 0.2 mg., calcium pantothenate 2 mg., inositol 10 mg., niacin 2 mg., *p*-aminobenzoic acid 25 mg., folic acid 0.025 mg., biotin 0.01 mg., choline 100 mg. per 100 gm. of ration. Vitamins A, D, and E were administered as a solution of 20 gm. of haliver oil, 18 gm. of corn oil, and 2 gm. of  $\alpha$ -tocopherol, at

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the rate of 2 drops per week. Sodium and potassium were added as indicated in Tables I, II, and III at the expense of the whole ration in the form of bicarbonates.

The sodium- and potassium-low Salts IV was obtained by removing the NaCl and  $K_2HPO_4$  from Salts IV (5). The phosphorus was maintained by substituting 505.4 gm. of  $CaHPO_4$  for 371.0 gm. of  $CaCO_3$ . The chloride was partially maintained by substituting 337.0 gm. of  $CaCl_2 \cdot 2H_2O$  for the balance of the  $CaCO_3$  (229.0 gm.). The resulting salt mixture, when fed at 2.5 per cent of the ration, supplied the same amounts of all

TABLE I  
*Effect of Dietary Sodium on Alloxan Diabetes*

K <sup>+</sup>  <i>per cent</i>	Na <sup>+</sup>  <i>per cent</i>	No. of animals	No. diabetic at	
			2 days	4 days
0.25	0.005	9	6 (5)*	
0.25	0.05	8	1	1
0.25	0.1	7	4	0
0.25	0.15	8	3	2
0.25	0.2	6	1	0
0.25	0.25	8	3	2
0.5	0.005	8	5	7 (2)
0.5	0.05	7	2	1
0.5	0.1	10	5	0
0.5	0.15	7	1	1
0.5	0.2	5	0	0
0.5	0.25	17	2	2

\* The number enclosed in parentheses represents the number of rats which died.

elements except chloride,<sup>1</sup> sodium, and potassium as Salts IV, fed at 4 per cent of the ration.

Male weanling rats (Holtzman), weighing 40 to 45 gm., were placed on the experimental diet for 10 to 12 weeks, at which time they were given, after a 15 hour fast, a subcutaneous injection of a 5 per cent aqueous solution of alloxan monohydrate (Pfanstiehl) at the rate of 75 mg. per kilo of body weight. Blood sugar was determined according to the method of Folin and Malmros as modified by Horvath and Knehr (6), following a 12 hour fast at 0, 2, and 4 days after the injection of alloxan.<sup>2</sup> Desoxy-

<sup>1</sup> The chloride which was present at 0.33 per cent of the ration was low; however, it was above the minimum requirement of 0.05 per cent (14).

<sup>2</sup> Those rats whose fasting blood sugars were greater than 175 mg. per cent were considered diabetic.

corticosterone acetate<sup>3</sup> (DCA) in peanut oil (4 mg. per cc.) was administered by subcutaneous injection at the rate of 2 mg. per rat per day. The rats weighed 135 gm. and were fed the basal ration plus 0.25 per cent sodium and 0.5 per cent potassium. Control rats were injected with an equal amount of peanut oil containing no DCA. Following 25 days of

TABLE II  
*Effect of Dietary Potassium on Incidence of Alloxan Diabetes*

Na <sup>+</sup>	K <sup>+</sup>	No. of animals	No. diabetic at	
			2 days	4 days
<i>per cent</i>	<i>per cent</i>			
0.005	0.1	8	7 (4)*	6
0.005	0.25	9	6 (5)	
0.005	0.5	8	5	7 (2)
0.005	1.0	10	7 (2)	6 (1)
0.1	0.25	7	4	0
0.1	0.5	10	5	0
0.25	0.25	8	3	2
0.25	0.5	17	2	2
0.25	5.0	8	2	0

\* The number enclosed in parentheses represents the number of rats which died.

TABLE III  
*Effect of Dietary Sodium on Blood Glutathione of Rats*

K <sup>+</sup>	Na <sup>+</sup>	No. of animals	Blood glutathione	
				Range
<i>per cent</i>	<i>per cent</i>		<i>mg. per cent</i>	<i>mg. per cent</i>
0.5	0.005	4	8.1	7.6-8.8
0.5	0.01	5	9.3	7.7-14.0
0.5	0.03	4	24.6	18.4-29.6
0.5	0.05	4	25.4	18.4-31.2
0.5	0.10	5	31.2	25.2-35.2

DCA treatment the rats were injected subcutaneously, after a 15 hour fast, with 100 mg. per kilo of alloxan. After a 4 day interval the rats not diabetic in the control group, and an equal number of DCA-treated rats, were injected after a 15 hour fast with a second dose of 125 mg. per kilo. Blood sugar was determined as described.

Blood glutathione was determined in the rats which were on the experi-

<sup>3</sup> Generously supplied through the courtesy of Roche-Organon, Inc., Nutley, New Jersey.

mental diet for 6 to 7 weeks, according to the method of Fujita and Numata as modified by Brückmann and Wertheimer (7). The procedure was further modified to adapt it to the use of the Evelyn colorimeter. It was necessary to double the reagent volumes and to add sufficient 2 per cent metaphosphoric acid in saturated sodium chloride to make a final volume of 10 cc. Contrary to Brückmann and Wertheimer, Beer's law did not apply, although a suitable standard curve was obtained. Since the color faded very rapidly and more so at low concentrations, it was necessary to make readings at 15 seconds to obtain acceptable analytical results on duplicate samples.

### *Results*

The level of alloxan required to produce diabetes in 90 to 95 per cent of the rats by subcutaneous injection after a 48 hour fast had been shown by Kass and Waisbren (8) to be 175 mg. per kilo. In order to demonstrate the increased susceptibility of sodium-deficient rats, the amount of alloxan had to be reduced to that concentration which would just fail to produce diabetes in the normal rat. The level was found to be 75 mg. per kilo. Of twelve rats which received 75 mg. per kilo after a 15 hour fast only one was diabetic on the 2nd day after the injection. When 100 mg. per kilo were used, nine out of twelve rats were diabetic on the 2nd day. The 75 mg. per kilo dose was used to test the increased susceptibility of sodium-deficient rats and the 100 mg. per kilo dose to test the protective effect of DCA.

The data in Table I indicate that when sodium was not added to the basal ration containing 0.25 per cent potassium (0.26 per cent sodium and 0.56 per cent potassium are present when Salts IV is fed at 4 per cent of the ration) the incidence of alloxan diabetes was greater than when 0.05 per cent or more sodium was present. Of the nine animals receiving no sodium, six were diabetic on the 2nd day after the injection, five of which died. When the sodium was increased to 0.05 per cent, the incidence was lowered to one out of eight. When 0.1 per cent sodium was present in the ration, the incidence increased to four out of seven, all of which returned to normal by the 4th day. Further increases of sodium up to 0.25 per cent resulted in no increased protection.

When potassium was present at 0.5 per cent, similar results were obtained. In the absence of added sodium, five out of eight rats were diabetic on the 2nd day. The number increased to seven on the 4th day, at which time two rats died. The presence of 0.05 per cent sodium reduced the incidence to two out of seven on the 2nd and one out of seven on the 4th day. 0.1 per cent sodium resulted in five out of ten becoming diabetic on the 2nd day, all of which returned to normal by the 4th day. No further



improvement was noticed when graded increments of sodium were fed up to 0.25 per cent.

The effect of potassium on the incidence of alloxan diabetes is summarized in Table II. When the potassium in the ration was increased from 0.1 to 1.0 per cent in the absence of added sodium, the incidence of diabetes was not affected. The severity of the diabetes as judged by the number of deaths was slightly lower when 0.5 and 1.0 per cent potassium was fed in the ration. When the potassium was doubled in the presence of 0.1 per cent sodium and increased 20-fold in the presence of 0.25 per cent sodium, no effect on the incidence of alloxan diabetes was noted. These data indicated that potassium at the levels used exerted little influence on the incidence of the disease.

When the amounts of sodium and potassium were increased to very high levels, the number of diabetic rats increased slightly, regardless of which ion was in excess. 2.5 per cent sodium and 0.25 per cent potassium resulted in three out of eight rats becoming diabetic by the 2nd and also the 4th day. 0.25 per cent sodium and 5.0 per cent potassium resulted in only two out of eight being diabetic on the 2nd day, both of which returned to normal on the 4th day. The presence of 1.0 per cent sodium and 2.0 per cent potassium resulted in four out of eight rats being diabetic on the 2nd day, while 2.0 per cent sodium and 1.0 per cent potassium resulted in a similar incidence. Comparison of these data with the control group (0.25 per cent sodium and 0.5 per cent potassium), which had only two diabetic rats out of seventeen, indicated that the presence of large amounts of sodium or potassium resulted in an increased incidence.

In agreement with Schrader *et al.* (9), rats fed a ration deficient only in potassium died rapidly, within 2 to 3 weeks. This prevented the determination of the direct effect of a potassium deficiency on the incidence of alloxan diabetes. Ferrebee *et al.* (10) among others had reported that desoxycorticosterone (DCA) would produce a state of potassium deficiency by increasing the excretion of potassium and decreasing that of sodium. Of the six rats which received DCA, none were diabetic after the first or second injection, while in the control group receiving no DCA, two were diabetic after the first injection and two more were diabetic after the second injection, or a total of four out of six rats. The failure of the DCA-treated rats to respond to the two doses of alloxan, while four out of six controls did respond, indicated that the hormone exerted a protective effect against alloxan diabetes.

The ability of glutathione to inhibit the action of alloxan (11) suggested that the increased susceptibility of sodium-deficient rats to alloxan might be explained by a lowered concentration of blood glutathione in the deficient animals. That this was the case is shown by the data in Table III. In

the absence of added sodium the level of blood glutathione was only 8.1 mg. per cent compared to 31.2 mg. per cent when 0.1 per cent sodium was present in the ration. When 0.05 per cent sodium was present, the level of blood glutathione was almost normal (the normal level for the rat is 30 to 45 mg. per cent (7)). The increased concentration of the blood glutathione as the sodium in the ration was increased from 0.005 to 0.1 per cent was correlated with the decrease in the incidence of alloxan diabetes, as shown in Table I. When the same levels of sodium were fed to the rats receiving only 0.25 per cent potassium, similar results were obtained.

#### DISCUSSION

The protective effect of sodium against alloxan diabetes is indicated by the decrease in the incidence of diabetic rats on the addition of sodium to the sodium-low basal ration. Since sodium-deficient rats had low concentrations of blood glutathione (Table III), a possible explanation of the effect of sodium would be as follows. Alloxan diabetes can be prevented by the simultaneous injection of glutathione (11), probably because of the complex formed between the two compounds (12). The presence of insufficient glutathione in the blood of sodium-deficient rats would permit more alloxan to reach the pancreas before being tied up as the complex, and, hence, the incidence would be greater than if normal concentrations of blood glutathione were present. The protective effect of sodium in concentrations as low as 0.05 per cent was correlated with the almost normal level of blood glutathione.

Whether or not the lowered glutathione level in the blood is specific for a sodium deficiency cannot be stated at this time. However, it should be noted that Binet and Pautonnet (13) reported lower levels of glutathione in the blood of patients suffering from Addison's disease and that DCA administration resulted in a return to normal. Normal hematocrit and hemoglobin values were obtained for the sodium-deficient animals, which indicated that the lowered glutathione level was not due merely to a lack of red blood cells or anemia.

The protective effect of DCA would be expected, since an excessive amount of the hormone is known to increase the excretion of potassium and decrease that of sodium (10). The resulting imbalance of sodium and potassium in the body which is the reverse of that obtained in a sodium deficiency would account for the protection by DCA. In this connection, it should be mentioned that Laszt (15) was able to "cure" six out of twenty-two alloxan-diabetic rats by the injection of DCA glucoside.

That various levels of potassium in the diet did not alter the incidence of diabetes is contrary to the reported antagonism of sodium and potassium

(1, 3). However, for excessive amounts of potassium to affect the electrolyte balance in the body the level of sodium in the diet must be low (unpublished report). In the presence of adequate sodium, excess potassium did not have any effect on the electrolyte balance. Thus the lack of evidence of potassium antagonism in these experiments does not appear unexpected.

In all groups studied the fasting level of blood sugar immediately prior to the injection of alloxan was constant at 90 to 105 mg. per cent regardless of the diet. This indicated that the varying amounts of sodium and potassium did not have any direct effect on carbohydrate metabolism as judged by the fasting level of blood sugar. It would seem therefore that the effect of a sodium deficiency on alloxan diabetes was due only to the lowering of the blood glutathione and not to a direct effect on carbohydrate metabolism.

#### SUMMARY

The effect of sodium and potassium on the induction of alloxan diabetes in the rat has been investigated. A sodium deficiency was found to result in an increased incidence of diabetes in the rat. Potassium was not antagonistic to sodium under the conditions used, but desoxycorticosterone did protect the rat against alloxan. The influence of the sodium deficiency may be explained by the low level of blood glutathione that was found in these animals.

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# STUDIES OF ACETOACETATE FORMATION WITH LABELED CARBON\*

## I. EXPERIMENTS WITH PYRUVATE, ACETATE, AND FATTY ACIDS IN WASHED LIVER HOMOGENATES

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Lehninger (1) has described the preparation of a liver homogenate which converts pyruvate or fatty acids into acetoacetate in good yield and which consumes little or no oxygen and does not produce acetoacetate endogenously.

The parallel behavior of octanoate and pyruvate in this preparation with regard to acetoacetate formation and the suppression of this process by the addition of members of the tricarboxylic acid cycle (1) emphasize the question of whether pyruvate and fatty acids give rise to identical intermediates in the process of forming acetoacetate or tricarboxylic acids. In the experiments reported here an attempt has been made by use of substrates labeled with  $C^{13}$  or  $C^{14}$  to determine whether or not pyruvate and octanoate give rise to a common intermediate in the process of forming acetoacetate and whether, if different, these intermediates can interact to produce acetoacetate of dual origin.

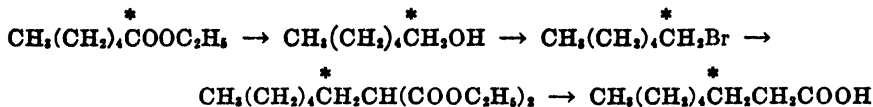
It was also felt worth while to repeat, in this system, the experiments of Weinhouse *et al.* (2) and Buchanan *et al.* (3) on the conversion of carboxyl-labeled octanoate to acetoacetate and also to study the distribution of isotope in the acetoacetate formed from isotopically labeled pyruvate.

### EXPERIMENTAL

*Organic Syntheses*—Acetate labeled with  $C^{13}$  in the carboxyl position was prepared from  $NaC^{13}N$  by the Walden (4) reaction. Pyruvate containing  $C^{13}$  or  $C^{14}$  in the  $\alpha$  and  $\beta$  positions was in some instances synthesized by the method previously reported by Sakami, Evans, and Gurin (5); it was also prepared from doubly labeled acetate by way of acetyl bromide, pyruvonitrile, and pyruvamide (6). Carboxyl-labeled octanoic and hexanoic acids were prepared from  $NaC^{13}N$  and the appropriate alkyl bro-

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mides. After hydrolysis of the resulting nitriles, the acids were distilled *in vacuo*. For the preparation of octanoic acid labeled in the  $\beta$  position (see the accompanying diagram), carboxyl-labeled hexanoic acid was



converted to the ethyl ester and reduced with  $\text{LiAlH}_4$  to hexyl alcohol. The alcohol was converted to hexyl bromide and condensed with ethyl malonate (7). After saponification and decarboxylation of the resulting product,  $\beta$ -labeled octanoic acid was separated and purified by distillation.

*Other Compounds*—Adenosine triphosphate (ATP) was prepared by the method of Kerr (8) as modified by Polis and Meyerhof (9). It was also obtained commercially as the tetrasodium salt from the Rohm and Haas Company. Crystalline sodium pyruvate was prepared from redistilled pyruvic acid which was dissolved in a minimal quantity of water, neutralized with sodium carbonate, evaporated *in vacuo* until crystallization began, and treated with small amounts of ethyl alcohol to induce further crystallization. This product was then recrystallized from water and ethyl alcohol. *N*-Octanoic acid was obtained from the Eastman Kodak Company. Malonic acid was purified via the calcium salt (10).

*Preparation and Incubation of Homogenates*—Washed homogenates of rat liver were prepared in a cold room ( $3^\circ$ ) according to the procedure described by Lehninger (11) with the slight modifications mentioned below. Since large quantities of homogenate were needed, a 100 ml. glass Potter-Elvehjem homogenizer was used. In order to obtain active preparations with this unusually large homogenizer it was necessary to run it, previously chilled, at 90 R.P.M. and not to attempt a complete excursion of the plunger without intermittent chilling of both plunger and cylinder. After one excursion of the plunger, the crude homogenate thus obtained was filtered through gauze, distributed among a sufficient number of 15 ml. conical centrifuge tubes, chilled again, and centrifuged for 7 minutes at 4000 R.P.M. in an angle head centrifuge. Although in Lehninger's original procedure the crude homogenate was centrifuged and resuspended four times, we found that the last two washings could be omitted without changing the character of the homogenate.

The homogenates were incubated in a medium essentially that of Lehninger (11). The incubation mixture consisted of one-third by volume of the washed homogenate and contained phosphate buffer, pH 7.7, 0.0085 M,  $\text{MgSO}_4$  0.0053 M, and ATP 0.0021 M. Malonate was included in the medium at a final concentration of 0.01 M except in the experiments

involving octanoic and hexanoic acids. The concentrations of substrates are listed in Tables I to V. The substrates were added to the medium prior to the addition of cold homogenate in an attempt to preserve the activity of the enzyme systems.

In order to obtain sufficient amounts of acetoacetate for chemical degradation and isotopic analysis, 75 ml. of combined homogenate and medium were incubated in each experiment. The gas phase was air. It was found that rapid shaking of the incubation flask inactivated the fatty acid oxidase system so that, in order to minimize the amount of shaking necessary for oxygen diffusion, the 75 ml. of fluid were equally divided among four 125 ml. Erlenmeyer flasks which contained alkali insets and which were tightly stoppered. The flasks were then shaken gently in a water bath at 25° until acetoacetate formation reached a limit.

In order to follow the course of the reaction, small aliquots of the reaction mixture were incubated simultaneously in Warburg manometer vessels. The relative shaking rates of the manometer vessels and the Erlenmeyer flasks had been previously adjusted so that the oxygen uptake of the manometer vessels gave indication of the extent of oxidation taking place in the large flasks. At the end of the incubation, the contents of the flasks were combined and small aliquots withdrawn for the determination of pyruvate or acetoacetate. Acetoacetate was determined manometrically by the method of Edson (12) and pyruvate by the method of Friedemann and Haugen (13).

The bulk of the homogenate was then divided into two portions. From approximately two-thirds of it  $\text{CO}_2$ , collected as  $\text{BaCO}_3$ , was obtained from the carboxyl group of acetoacetate by a procedure previously described, involving treatment with aniline citrate (3). The mercury-acetone complex derived from the acetoacetate was obtained from a copper-lime filtrate prepared from the remaining third of the homogenate (3).

The  $\text{BaCO}_3$  was analyzed for  $\text{C}^{13}$  by a mass spectrometer<sup>1</sup> or for  $\text{C}^{14}$  in a Geiger counter (14). The mercury-acetone complex was oxidized to  $\text{CO}_2$  and water, and this  $\text{CO}_2$  was analyzed for  $\text{C}^{13}$  or  $\text{C}^{14}$ .

Since it has been previously shown that all of the isotope in the acetoacetate formed from carboxyl-labeled octanoate is located in the carbonyl and carboxyl carbons of acetoacetate (2), we calculated the concentration of isotope in the carbonyl carbon by multiplying the value obtained for acetone by 3 for all acetoacetate derived from monolabeled fatty acids.

A few degradations of acetoacetate were carried out by the heat decarboxylation of copper-lime filtrates prepared from entire homogenates. Here, liberation of carboxyl  $\text{CO}_2$  and the formation of the mercury-acetone

<sup>1</sup> We are indebted to Dr. Sidney Weinhouse and the Sun Oil Company for these analyses.

complex were accomplished simultaneously by heating the  $\text{CO}_2$ -free filtrate to  $100^\circ$  in the presence of Denigès' reagent.

### Results

*Conversion of  $\alpha,\beta$ -Labeled Pyruvate to Acetoacetate*—The formation of acetoacetate from pyruvate by liver and other tissues is a well established metabolic reaction (15–17). It has been generally assumed that an acetoacetate molecule arises from the acetyl portions of 2 pyruvate molecules whose carboxyl carbons have been eliminated as  $\text{CO}_2$ .

If only the acetyl carbons of pyruvate are utilized in this reaction, incubation of  $\alpha,\beta$ -labeled pyruvate in this system would be expected to give rise to acetoacetate containing a concentration of isotope in each of its

TABLE I  
*Acetoacetate Formation from Pyruvate Containing  $\text{C}^{13}$  or  $\text{C}^{14}$  in  $\alpha$ - and  $\beta$ -Carbons*

Experiment No.	Initial concentration of pyruvate in medium	Pyruvate utilized	Final total acetoacetate	Atom per cent excess $\text{C}^{13}$ or counts per mg. carbon per min. for		
				$\alpha$ - and $\beta$ -carbons of added pyruvate	Final acetoacetate	
					$\text{CH}_3\text{C}(=\text{O})\text{CH}_2-$	$-\text{COOH}$
	M	$\mu\text{M}$	$\mu\text{M}$			
1	0.0120	244	137		1.34†	1.37†
2A	0.0222	545	176	56	27‡	32‡
2B	0.00		0			
3A	0.0112	735	238	711	460‡	455‡
3B	0.00		0			

†  $\text{C}^{13}$  used. In this experiment the respiratory  $\text{CO}_2$  contained 0.07 atom per cent  $\text{C}^{13}$ .

‡  $\text{C}^{14}$  used.

carbons equal to the concentration of isotope in the acetyl carbons of the added pyruvate. Furthermore, the carbon dioxide formed in the reaction should contain no isotope, since the carboxyl group of the added pyruvate contained none.

The experiments (Table I) show that the concentrations of isotope in the acetone and carboxyl carbons of the acetoacetate formed in the homogenate were essentially equal, indicating that all 4 carbons of the acetoacetate contained an equal concentration of isotope. In Experiment 1, metabolic carbon dioxide obtained from the incubation mixture contained little more than a trace of isotope. Since in this preparation  $\text{CO}_2$  does not arise endogenously (11), this  $\text{CO}_2$  probably arose from the unlabeled carbon of pyruvate. These results are in agreement with the conclusion that the  $\alpha,\beta$ -carbons of pyruvate contribute equally to both halves of acetoacetic



acid, while the carboxyl carbon is lost as  $\text{CO}_2$ . Further evidence for this conclusion is provided by the formation of unlabeled acetoacetate when carboxyl-labeled pyruvate was incubated with rat liver slices.<sup>2</sup>

In Experiments 2A and 3A a comparison of the concentrations of isotope in the acetyl carbons of the added pyruvate with that of the carbons of acetoacetate shows that an almost 2-fold dilution of isotope occurred during this conversion. The nature of this dilution is unexplained. That these particular homogenates did not produce or contain acetoacetate in the absence of added substrate is shown in control Experiments 2B and 3B. Except in the case of Experiment 1, the disappearance of pyruvate was more than adequate to account for the acetoacetate formed. In contrast to this, a relatively slight dilution of isotope was observed in the acetoacetate formed from isotopically labeled fatty acids (Table IV).

*Conversion of Carboxyl-Labeled Acetate to Acetoacetate*—Preliminary analytical studies in this laboratory showed that although, in agreement with Lehninger (11), acetate alone will not form acetoacetate in this homogenate, when supplemented with pyruvate it gave slight but reproducible increases in the yield of acetoacetate. An attempt to determine the extent of incorporation of acetate carbon into the two halves of acetoacetate was made by incubating carboxyl-labeled acetate and unlabeled pyruvate together in the homogenate.

The results (Table II) show that in the presence of unlabeled pyruvate a small but significant incorporation of acetate carbon into acetoacetate occurred, and that within the limits of experimental error the isotope was equally distributed between the carbonyl and carboxyl positions of the acetoacetate. On the average, 5 per cent of the total acetoacetate formed arose from labeled acetate. The amount of incorporation of isotope was independent of the concentration of acetate in the medium in the range studied (0.020 to 0.001 M).

A possible interpretation of these results is that amounts of acetoacetate, too small to be detected by the analytical methods used, are actually formed from acetate, thus accounting for the small concentration of isotope found in the acetoacetate of Experiments 1, 2, 3A, and 4A (Table II). This possibility was eliminated, however, by the control Experiments 3B and 4B in which portions of the same homogenates used in Experiments 3A and 4A, respectively, were incubated with carboxyl-labeled acetate alone. An amount of unlabeled acetoacetate comparable to that formed from pyruvate in Experiments 3A and 4A was mixed with the homogenate either before or after the incubation, and at the end of the incubation it was decomposed into acetone and  $\text{CO}_2$  for isotopic analysis. Here the traces of isotope found were relatively insignificant, indicating that the

<sup>2</sup> A personal communication from Dr. John M. Buchanan.

conversion of acetate to acetoacetate depended entirely on pyruvate in these experiments.

Similar experiments were carried out with carboxyl-labeled acetate and unlabeled octanoate as the active cosubstrate. In these experiments (Table III) it was not possible to include malonate in the medium, since washed homogenates of the liver of rats of the Wistar strain do not oxidize octanoate in the presence of malonate. These homogenates were highly active in the absence of malonate, but, unlike Lehninger's prepara-

TABLE II

*Incorporation of Isotope in Acetoacetate Derived from Carboxyl-Labeled Acetate in Presence of Non-Labeled Pyruvate*

Experiment No.	Initial concentrations of substrates in medium		Acetoacetate formed	Acetoacetate added	Total acetoacetate	Atom per cent excess C <sup>13</sup> in total acetoacetate		Distribution of C <sup>13</sup> in acetoacetate	Acetate converted to acetoacetate† (calculated)
	Carboxyl-labeled acetate‡	Pyruvate				$\overset{\circ}{\text{C}}\text{O}$ (B)	$\overset{\circ}{\text{C}}\text{OOH}$ (C)		
	M	M	$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$	(A)		$\frac{\overset{\circ}{\text{C}}\text{O}}{\overset{\circ}{\text{C}}\text{OOH}}$	$\mu\text{M}$
1	0.020	0.015	264	0	264	0.21	0.23	0.91	19.5
2	0.006	0.015	195	0	195	0.36	0.39	0.92	24.4
3A	0.006	0.015	185.5	0	186	0.39	0.35	1.11	22.9
3B	0.006	0	(1.1)	212§	213	0.03	0.03		2.1
4A	0.001	0.008	276	0	276	0.30	0.30	1.00	27.6
4B	0.001	0	(0.8)	163	164	0.03	0.03		1.6

† Acetoacetate derived from acetate=

micromoles total acetoacetate (atom % excess C<sup>13</sup> in carboxyl + carbonyl carbons)

atom % excess C<sup>13</sup> in carboxyl carbon of acetate

Column A × Columns B + C

atom % excess C<sup>13</sup> in carboxyl carbon of acetate

‡ The carboxyl carbon contained 6.0 atom per cent excess C<sup>13</sup>.

§ Added after incubation period prior to degradation of acetoacetate into acetone and CO<sub>2</sub>.

|| Added at beginning of incubation period.

tions, exhibited a slight residual oxygen uptake and were capable of converting acetate to acetoacetate to an extremely small but variable extent. No acetoacetate was formed in the absence of added substrates. It was also observed that frequently concentrations of acetate above 0.001 M partially inhibited the oxidation of octanoate.

Experiments 1, 2, and 3 (Table III) show that in the presence of non-labeled octanoate a 2- to 6-fold increase in the conversion of labeled acetate to acetoacetate occurred. Only 6 to 10 per cent of the total acetoacetate formed arose from labeled acetate. The distribution of C<sup>13</sup> between the

carbonyl and carboxyl positions of the acetoacetate was significantly uneven and when Experiments 1A and 2A were compared with their respective controls, Experiments 1B and 2B, which were run on portions of the same homogenate in each case, it was possible to calculate, as shown in Table III, the distribution of isotope between the carbonyl and carboxyl positions of the increment of isotopic acetoacetate whose formation depended upon the simultaneous oxidation of unlabeled octanoate. The

TABLE III

*Incorporation of Isotope in Acetoacetate Derived from Carboxyl-Labeled Acetate in Presence of Non-Labeled Octanoate*

Ex- peri- ment No.	Initial concen- trations of substrates in medium		Aceto- acetate formed	Aceto- acetate added initially	Total aceto- acetate  (A)	Atom per cent excess C <sup>13</sup> in total aceto- acetate		Acetate converted to acetoacetate† (calculated)		Distribution of acetate carbon in acetoacetate  $\frac{\text{CH}_3\text{CO}-}{-\text{CH}_2\text{COOH}}$
	Carboxyl- labeled acetate‡	Octa- noate				$\overset{\circ}{\text{C}}\text{O}$ (B)	$\overset{\circ}{\text{C}}\text{OOH}$ (C)	$\text{CH}_3\overset{\circ}{\text{C}}\text{O}-$ (D)	$-\text{CH}_2\overset{\circ}{\text{C}}\text{OOH}$ (E)	
	M	M	$\mu\text{M}$	$\mu\text{M}$	$\mu\text{M}$			$\mu\text{M}$	$\mu\text{M}$	
1A	0.002	0.001	49.1	0	49	0.27	0.51	2.2	4.2	0.47§
1B	0.002	0	(0.4)	227	227	0.01	0.01	0.4	0.4	
								1.8§	3.8§	
2A	0.004	0.001	163	0	163	0.51	0.66	13.9	18.0	0.58§
2B	0.004	0	(8)	215	223	0.21	0.20	7.8	7.4	
								6.1§	10.6§	
3A	0.001	0.001	136	0	136	Lost	0.57		12.9	
3B	0.001	0	(2)	165	167	"	0.07		2.0	

† Calculation of micromoles of acetate converted to  $\text{CH}_3\text{CO}-$  or  $-\text{CH}_2\text{COOH}$  of acetoacetate =

$$\text{micromoles} = \frac{\text{Column A} \times \text{Column B or C}}{\text{atom \% excess C}^{13} \text{ in carboxyl carbon of acetate (6.0)}}$$

‡ Carboxyl carbon contained 6.0 atom per cent excess C<sup>13</sup>.

§ These figures concern the increment of acetate carbon whose incorporation into acetoacetate is due to the presence of non-labeled octanoate.

\* \*  
CO:COOH ratios\* for these increments show that approximately twice as much acetate carbon was incorporated in the carboxyl half of the acetoacetate as in the carbonyl half. This indicates that at least 50 per cent of this labeled acetoacetate must have been purely carboxyl-labeled and therefore must have arisen as a result of the acetylation of carboxyl-labeled acetate (or an active derivative) by non-labeled 2-carbon compounds formed by octanoate.

\* To be used henceforth to designate the ratio of the concentration of labeled carbon in the carbonyl position to that in the carboxyl position of acetoacetate.

The incubation of carboxyl-labeled acetate with liver slices has been reported by Weinhouse, Medes, and Floyd (18) to result in the formation of asymmetrically labeled acetoacetate with a  $\text{CO}^*: \text{COOH}^*$  ratio of 0.81, suggesting that in the liver slice the endogenous oxidation of fatty acids exerted a similar influence on the conversion of acetate to acetoacetate.

*Conversion of Labeled Fatty Acids to Acetoacetate*—In the experiments of Weinhouse, Medes, and Floyd (2) the incubation of carboxyl-labeled octanoate with rat liver slices resulted in the formation of labeled acetoacetate, with an equal distribution of the isotope between the carbonyl and carboxyl carbons. These results, considered together with the evidence reported by Buchanan, Sakami, and Gurin (3) that carboxyl-labeled acetoacetate is not converted to symmetrically labeled acetoacetate by rat liver slices, are in accord with the theory proposed by MacKay *et al.* (19) that fatty acids are completely converted into a *single species* of 2-carbon units which undergo random condensation to form acetoacetate.

In an attempt to repeat the experiments of Weinhouse *et al.* (2), Buchanan *et al.* (3), using identical conditions, obtained labeled acetoacetate from carboxyl-labeled octanoate with a  $\text{CO}^*: \text{COOH}^*$  ratio of 0.65. These authors considered this asymmetrically labeled acetoacetate to have arisen partially by multiple alternate oxidation. In view of the experiments reported in this paper another explanation seems more likely.

When carboxyl-labeled octanoate was incubated alone in the washed liver homogenate, the distribution of the isotope in the resulting acetoacetate was highly unequal, a large excess of isotope appearing in the carboxyl position (Table IV, Experiments 1, 2, 3A, 4A, 5A, 5B, and 6). The average  $\text{CO}^*: \text{COOH}^*$  ratio was 0.66. Similar results were obtained with carboxyl-labeled hexanoate (Experiments 10 and 11).

In each of these experiments the dilution of isotope accompanying the conversion of the labeled fatty acid to acetoacetate was calculated by comparing the average concentration of  $\text{C}^{13}$  in the carbons of acetoacetate with the average concentration of  $\text{C}^{13}$  in the carbons of the added mono-labeled fatty acid. It was found that the acetoacetate contained, on the average, 85 per cent of the over-all  $\text{C}^{13}$  concentration of the original fatty acid; the highest value observed was 94 per cent. The figures indicate either slight dilution by endogenous acetoacetate formation or perhaps slight contamination of the mercury-acetone fraction, which will be discussed below. Whatever the cause, the dilution is relatively small in comparison with the dilution of isotope observed in the acetoacetate formed from isotopically labeled pyruvate (Table I) in which only 61 per cent of the expected concentration of isotope was found in the acetoacetate.

If the appearance of more  $C^{13}$  in the carboxyl than in the carbonyl position of the acetoacetate was due to the occurrence of some multiple alternate oxidation of the carboxyl-labeled octanoate, then  $\beta$ -labeled octanoate should give rise to acetoacetate with an equal preponderance of

TABLE IV

*Acetoacetate Formation in Washed Homogenate of Rat Liver Incubated with Carboxyl-Labeled Fatty Acids Alone and in Presence of Non-Labeled Pyruvate*

Experiment No.	Substrates	Atom per cent excess $C^{13}$ in carboxyl carbon	Initial concentration of substrate in medium	Final acetoacetate	Atom per cent excess $C^{13}$ in final acetoacetate		Distribution of $C^{13}$ in final acetoacetate
					CO	COOH	
			M	$\mu M$			$\frac{CO}{COOH}$
1	Octanoate	7.68	0.0010	117	1.32	2.01	0.66
2	"	7.68	0.0010	144	1.47	1.79	0.82
3A†	"	23.8	0.0010	161	3.72	6.64	0.56
4A†	"	23.8	0.0010	156	4.17	6.07	0.69
5A‡	"	23.8	0.0010	75	3.45	6.05	0.57
5B‡	"	23.8	0.0010	199	3.84	5.92	0.65
6§	"	23.8	0.0010	253	4.51	6.54	0.69
7	Octanoate	7.68	0.0010	250	0.84	0.84	1.00
	+ pyruvate	None	0.0040				
8	Octanoate	7.68	0.0010	258	0.87	1.01	0.86
	+ pyruvate	None	0.0040				
3B†	Octanoate	23.8	0.0010	112	1.62	1.99	0.81
	+ pyruvate	None	0.0080				
4B†	Octanoate	23.8	0.0010	223	1.86	2.28	0.82
	+ pyruvate	None	0.0080				
9§	Octanoate	23.8	0.0010	222	2.30	2.82	0.82
	+ pyruvate	None	0.0040				
10	Hexanoate	23.8	0.0015	134	4.95	8.48	0.58
11	"	23.8	0.0010	172	4.65	8.36	0.56

† Experiments A and B run on the portions of the same homogenate.

‡ Homogenate 3 times more concentrated in Experiment B than in Experiment A.

§ Heat degradation of acetoacetate; all others by aniline citrate.

$C^{13}$  in the carbonyl position (i.e., a  $\frac{CO}{COOH}$  ratio of 1.34). Accordingly,  $\beta$ -labeled octanoate was incubated in the washed homogenate (Table V, Experiments 1 and 2) and the resulting acetoacetate was found to have an average  $\frac{CO}{COOH}$  ratio of 0.50, thus eliminating the possibility of multiple alternate oxidation as a significant factor in these experiments. Although this ratio is slightly lower than the average value

obtained in the experiments with carboxyl-labeled octanoate, this difference can be partially attributed to the rather low yields of acetoacetate obtained in the experiments with  $\beta$ -labeled octanoate. Errors accompanying low yields of acetoacetate are discussed below.

The fatty acid oxidase activity of the different homogenates used in these experiments varied several fold and the possibility that variations in the  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratio of acetoacetate might be related to the speed of fatty acid oxidation was tested in Experiments 5A and 5B (Table IV). Here two portions of the same homogenate with tissue concentrations adjusted to give a 3-fold difference in the rates of oxygen consumption in the presence of 0.001 M carboxyl-labeled octanoate were compared. The

TABLE V

*Acetoacetate Formation in Washed Homogenate of Rat Liver from  $\beta$ -Labeled Octanoate Alone and in Presence of Non-Labeled Pyruvate*

Experiment No.	Substrates	Atom per cent excess of $\text{C}^{14}$ in $\beta$ -carbon	Initial concentration of substrate in medium	Final acetoacetate	Atom per cent excess $\text{C}^{14}$ in final acetoacetate		Distribution of $\text{C}^{14}$ in final acetoacetate
					$\overset{*}{\text{CO}}$	$\overset{*}{\text{COOH}}$	$\frac{\overset{*}{\text{CO}}}{\overset{*}{\text{COOH}}}$
1	Octanoate	4.8	0.0010	110	0.63	1.36	0.46
2	"	4.8	0.0010	103	0.63	1.19	0.53
3	Octanoate	4.8	0.0005	118	0.33	0.39	0.85
4	+ pyruvate	None	0.0040	192	0.48	0.55	0.87
	Octanoate	4.8	0.0005				
	+ pyruvate	None	0.0020				

$\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratios for acetoacetate formed by the diluted and concentrated homogenate were 0.57 and 0.65 respectively, suggesting that variations in the rate of fatty acid oxidation did not affect the distribution of isotope significantly.

In order to test the possibility that pyruvate and octanoate might interact to produce a composite species of acetoacetate, experiments were carried out in which either carboxyl-labeled octanoate (Table IV, Experiments 7, 8, 3B, 4B, and 9) or  $\beta$ -labeled octanoate (Table V, Experiments 3 and 4) was incubated together with unlabeled pyruvate. It was found in preliminary studies of such incubations that pyruvate and octanoate at concentrations of 0.004 and 0.001 M respectively (which are equivalent for acetoacetate production) were oxidized at maximal and nearly equal rates when incubated in portions of the same homogenate. The incubation of both substrates together in another portion of the same homoge-

nate resulted in an increased rate of oxygen consumption which was essentially equal to the sum of the rates obtained when the two substrates were incubated separately. Under these conditions it was assumed that both pyruvate and octanoate were being converted to acetoacetate simultaneously and that their intermediates had the opportunity to mingle and possibly interact.

In all of the isotope experiments of this type, a significantly higher  $^{14}\text{C}$ : $^{14}\text{C}$  CO:COOH ratio was observed than in experiments in which carboxyl- or  $\beta$ -labeled octanoate was incubated alone. The average  $^{14}\text{C}$ : $^{14}\text{C}$  CO:COOH ratio was 0.86 as compared with 0.63 for the acetoacetate formed from carboxyl- or  $\beta$ -labeled octanoate incubated alone. In Experiments 3 and 4 (Table IV) this comparison was made on different portions of the same homogenate.

The possibility that the discrepancy between the uniform distribution of  $\text{C}^{13}$  in the acetoacetate formed from carboxyl-labeled octanoate in the experiments of Weinhouse *et al.* (2) and the uneven distribution found consistently in this laboratory could be a technical artifact was critically examined during the later stages of this study.

Carbon dioxide and acetone were prepared from synthetic acetoacetate<sup>4</sup> containing equal concentrations of isotope in the carbonyl and carboxyl positions by both of the degradation procedures used in the biological experiments (described above). Furthermore, some of these degradations were carried out in copper-lime filtrates made from homogenates which had been incubated with and without non-labeled pyruvate or octanoate. It was found that in general a small but detectable contamination of the mercury-acetone occurred. The magnitude of this contamination appeared to be unaffected by the presence of copper-lime filtrates of homogenates during the degradation but rather to vary inversely with the total amount of acetone recovered (*e.g.* 7 per cent for the isolation of 162  $\mu\text{M}$  and 16 per cent for 73  $\mu\text{M}$  of acetone).

The effect of these errors upon the results obtained in the biological experiments reported here was estimated and found not to change them sufficiently to warrant the calculation of new values. Furthermore, they are insufficient to account for the difference between the previous results of Weinhouse *et al.* (2) and those obtained here on the distribution of  $\text{C}^{13}$  in the acetoacetate formed from carboxyl-labeled octanoate in liver tissue.

#### DISCUSSION

The data presented here (Table I) and previous related evidence (14, 20) leave little doubt that acetoacetate is derived from the acetyl groups of pyruvate. Whether these acetyl groups are converted into 2-carbon

<sup>4</sup> A gift from Dr. Sidney Weinhouse.

compounds in the process of forming acetoacetate is not known. Attempts to establish 5- or 6-carbon compounds as intermediates have, to date, been unsuccessful. Acetopyruvate, reported by Krebs (16) to form acetoacetate readily in liver slices, was found to be inactive in the washed liver homogenate (1). Parapyruvic acid, reported by Annau (21) to form acetoacetate in liver homogenate, was found in this laboratory to be inactive in the washed liver homogenate, as were itaconic, citraconic, and mesaconic acids. These fragmentary negative results, however, do not rule out the possibility that some 5- or 6-carbon intermediate exists.

Suggestive evidence that pyruvate gives rise to a 2-carbon precursor of acetoacetate is provided by the effect of pyruvate in inducing carboxyl-labeled acetate to form symmetrically labeled acetoacetate (Table II) in the washed liver homogenate. It is reasonable to suppose that the stimulatory effect of pyruvate involves the conversion of acetate into an active 2-carbon intermediate. From the symmetrical distribution of isotope in the mixed acetoacetate formed from the isotopically labeled "active" acetate and the non-isotopic intermediates formed from pyruvate, it would appear that these intermediates are capable of undergoing random condensation with each other and are, therefore, identical. This interpretation may be in conflict with the evidence of Bloch and Rittenberg (22), confirmed by Anker in rats of the Wistar strain (20), that pyruvate, unlike acetate, will not readily acetylate aromatic amines *in vivo*, a matter which will be discussed more fully in Paper II (23).

The interaction of carboxyl-labeled acetate with non-labeled octanoate to form asymmetrically labeled acetoacetate with an average  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratio of 0.53 (Table III) is an indication that octanoate differs from pyruvate in the production of an intermediate which preferentially acetylates acetate (or "active" acetate). Furthermore, the  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratio of this acetoacetate agrees within the limits of experimental error with the average values found for this ratio in the acetoacetate formed from carboxyl-labeled (Table IV) and  $\beta$ -labeled (Table V) octanoate, suggesting that acetate and the first 4 carbons of octanoate give rise to an identical 2-carbon precursor of acetoacetate.

The conversion, in this homogenate, of carboxyl- and  $\beta$ -labeled octanoate into acetoacetate with  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratios of 0.65 and 0.50, respectively, conclusively rules out multiple alternate oxidation as an explanation for this uneven distribution of isotope. A modification of the  $\beta$ -oxidation-condensation hypothesis (19), however, which will explain these results is that *two species* of 2-carbon fragments arise from a fatty acid, with the result that condensation between these fragments to form acetoacetate is not completely random. According to this mechanism,



asymmetrically labeled acetoacetate would result from the condensation of 2-carbon units derived from monolabeled fatty acids. This would imply that the 2-carbon fragment derived from the carboxyl and  $\alpha$ -carbons of octanoate (carboxyl fragment) and the fragment derived from the  $\beta$ - and  $\gamma$ -carbons ( $\beta$ -fragment) are preferentially acetylated by 2-carbon fragments arising from the remaining carbons of the fatty acid chain. In terms of this hypothesis, the  $\beta$ - and carboxyl fragments of octanoate are identical, since both  $\beta$ - and carboxyl-labeled octanoate give rise to acetoacetate, with essentially the same distribution of isotope. The fragment derived from the  $\delta$ - and  $\epsilon$ -carbons ( $\delta$  fragment) would be expected to be identical with the  $\beta$  fragment, since both arise from a structurally similar portion of the fatty acid chain. By elimination, the fragment derived from the  $\zeta$ - and  $\omega$ -carbons (terminal fragment) of octanoate should differ from the other three, and  $\zeta$ -labeled octanoate should therefore give rise to predominantly carbonyl-labeled acetoacetate. Experiments showing the conversion of  $\zeta$ -labeled octanoate into acetoacetate containing the distribution of isotope required by this hypothesis will be presented in Paper II (23).

According to this mechanism, shortening the chain of a carboxyl-labeled fatty acid should increase the relative importance of the terminal fragment and therefore lead to a reduction of the  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratio of the acetoacetate derived from it. Consequently one would expect a small difference between  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratios in experiments with carboxyl-labeled octanoate and hexanoate, whereas much larger differences in the values would be obtained by comparing carboxyl-labeled butyrate and octanoate. Our figures show an average value of 0.65 for the  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratio of acetoacetate derived from carboxyl-labeled octanoate as compared with 0.57 for acetoacetate derived from carboxyl-labeled hexanoate. This small difference, although in accord with theoretical expectations, is also within the limits of experimental error and therefore not significant. A comparison of carboxyl-labeled butyrate and carboxyl-labeled octanoate may be drawn from the experiments of Weinhouse *et al.* in which these substances were incubated with liver slices (2, 24). In these experiments, unlike those reported here, symmetrically labeled acetoacetate was formed from carboxyl-labeled octanoate, and under the same conditions carboxyl-labeled butyrate gave rise to acetoacetate with a  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratio of 0.58.

To account for the observation that the  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratio for acetoacetate produced by a mixture of either carboxyl- or  $\beta$ -labeled octanoate and unlabeled pyruvate was significantly greater than the ratio found in aceto-

acetate produced when either monolabeled fatty acid was incubated alone (Tables IV and V), it is assumed that pyruvate must have contributed more carbon to the carboxyl than to the carbonyl portion of acetoacetate. In this respect its action is similar to that of acetate and the carboxyl and  $\beta$  fragments of octanoate.

The formation of asymmetrically labeled acetoacetate from carboxyl-labeled octanoate in the experiments reported here and in previous experiments carried out with liver slices in this laboratory (3) does not in our opinion conflict with the experiments of Weinhouse *et al.* (2) in which symmetrically labeled acetoacetate was obtained from carboxyl-labeled octanoate in liver slices. It is quite possible that in the latter experiments the magnitude of endogenous pyruvate and long chain fatty acid catabolism was sufficiently large nearly to equalize the distribution of isotope in the final acetoacetate, in accord with the phenomena observed here.

We wish to thank Dr. Adelaide M. Delluva for analytical aid and to express our appreciation to Dr. D. Wright Wilson for his interest and advice.

#### SUMMARY

The incubation of  $\alpha,\beta$ -labeled pyruvate in washed homogenates of rat liver resulted in the formation of unlabeled carbon dioxide and of acetoacetate containing equal concentrations of isotope in the acetone and carboxyl carbons, in agreement with the conclusion that the  $\alpha$ - and  $\beta$ -but not the carboxyl carbons of pyruvate are used in the biosynthesis of acetoacetate.

Pyruvate and octanoate stimulated the conversion of acetate into acetoacetate in the washed homogenate. Octanoate, however, differed from pyruvate in the production of asymmetrically labeled acetoacetate from carboxyl-labeled acetate.

Carboxyl- and  $\beta$ -labeled octanoate both gave rise to acetoacetate containing more  $C^{13}$  in the carboxyl than in the carbonyl position, thus eliminating multiple alternate oxidation as a significant process in this pathway. In both instances, simultaneous incubation of the monolabeled fatty acid with non-labeled pyruvate partially equalized the distribution of the isotope between the two positions.

In the presence of octanoate, pyruvate and acetate showed a similar tendency to introduce their carbons preferentially into the carboxyl portion of acetoacetate and, in this respect, were similar to the 2-carbon fragments which arose from the first 4 carbons of octanoate.

A hypothesis that fatty acids give rise to more than one type of 2-carbon

intermediate in the process of forming acetoacetate is proposed as an explanation for the uneven distribution of isotope in the acetoacetate formed from both carboxyl- and  $\beta$ -labeled octanoate.

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# STUDIES OF ACETOACETATE FORMATION WITH LABELED CARBON\*

## II. THE CONVERSION OF $\gamma$ -(C<sub>7</sub>)-LABELED OCTANOATE TO ACETOACETATE

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In Paper I (1), data were reported showing that acetoacetate containing more isotope in the carboxyl than in the carbonyl position was formed from both carboxyl- and  $\beta$ -labeled octanoate by washed homogenate of rat liver. It was suggested that fatty acids give rise by successive  $\beta$  oxidation to more than one species of 2-carbon units, the recombination of which to form acetoacetate would not be completely random. According to this concept, octanoate labeled in the  $\gamma$ -carbon should give rise to acetoacetate labeled predominantly in the carbonyl position (1). In this paper experiments with  $\gamma$ -labeled octanoate are reported which are in accord with this hypothesis and the relationships between these results and those reported previously (1) are discussed.

### EXPERIMENTAL

*Organic Synthesis*—The preparation of  $\text{CH}_3\text{C}^{14}\text{H}_2(\text{CH}_2)_6\text{COOH}$  (Diagram 1) was accomplished by the condensation of ethyl adipyl chloride (2) with diethyl cadmium to form ethyl  $\epsilon$ -keto octanoate (3). The diethyl cadmium complex was prepared from  $\text{CH}_3\text{C}^{14}\text{H}_2\text{I}$  via the Grignard reagent.

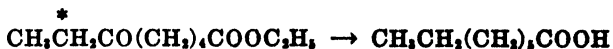


DIAGRAM 1

The resulting ethyl  $\epsilon$ -keto octanoate was reduced to the saturated acid and saponified simultaneously by a modification of the Wolff-Kishner method (4, 5). After working up the acid in the usual manner, the product was distilled at 108–109° at 3 mm. of mercury. The over-all yield for the two reactions was 68 per cent.

The identity of the product was confirmed by the preparation of the

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benzylthiuronium salt (m.p. 149° corrected). Upon mixing with an authentic sample, there was no depression of the melting point.

The washed homogenates of rat liver were prepared and incubated as previously described (1). The acetoacetate formed in the homogenates was decarboxylated and the acetone and CO<sub>2</sub> thus obtained were converted to BaCO<sub>3</sub> for measurement of radioactivity as previously described (1).

TABLE I

*Acetoacetate Formation in Washed Homogenate of Rat Liver Incubated with Isotopically Labeled Octanoate Alone and in Presence of Non-Labeled Pyruvate*

Experiment No.	Substrates			Final acetoacetate		
		Radioactivity of labeled carbon (counts per mg. C per min.)	Initial concentration in medium	Radioactivity (counts per mg. C per min.)		Distribution of C <sup>14</sup>
				CO	COOH	
1	*COOH octanoate	6600	0.0012	257	347	0.74
2	" "	6600	0.0010	251	336	0.75
3	†-Octanoate	6100	0.0010	395	110	3.6
4	" "	6100	0.0012	445	154	2.9
5	" "	6100	0.0010	414	119	3.5
6	†-Octanoate	6100	0.0010	432	136	3.2
	+ pyruvate	Not labeled	0.0040			
7	†-Octanoate	6100	0.0010	465	123	3.8
	+ pyruvate	Not labeled	0.0040			
8	†-Octanoate	6100	0.0010	411	135	3.0
	+ pyruvate	Not labeled	0.0080			

In all experiments an amount of non-labeled acetoacetate was added to the medium prior to the degradation so that the total quantity degraded was approximately 850 micromoles.

In these experiments, freshly prepared sodium acetoacetate was added to the reaction mixture at the end of the incubation period as carrier in order to provide sufficiently large quantities of acetoacetate for chemical degradation and measurement of radioactivity.

### Results

Experiments 1 and 2 (Table I) were included as controls to show that the distribution of C<sup>14</sup> in acetoacetate formed from carboxyl-labeled octanoate confirmed the results reported previously of identical experiments run with carboxyl C<sup>13</sup>-labeled octanoate (1). With C<sup>14</sup> a

$^{14}\text{C}$ : $^{12}\text{C}$  ratio<sup>1</sup> of 0.75 was obtained as compared with an average value of 0.65 found in the previous experiments with  $\text{C}^{13}$ . This small discrepancy is partly due to an increase in the purity of the mercury-acetone recovered from the homogenate, since larger yields of this compound were isolated in the present experiments than previously, and is partly due to a slight contamination of the  $\text{CO}_2$  fraction, since heat decarboxylation was employed here as compared with aniline citrate decarboxylation in the previous experiments.

In contrast to the previously reported experiments with carboxyl- and  $\beta$ -labeled octanoate (1), Experiments 3, 4, and 5 show that the  $^{14}\text{C}$ : $^{12}\text{C}$  ratio for the acetoacetate formed from  $\zeta$ -labeled octanoate averaged 3.3. Simultaneous incubation of the  $\zeta$ -labeled octanoate with non-isotopic pyruvate, which was previously shown to change this ratio from average values of 0.63 to 0.86 in experiments with carboxyl- and  $\beta$ -labeled octanoate (1), resulted here (Experiments 6, 7, and 8) in an average value of 3.3. It may therefore be concluded that pyruvate has no effect on the final distribution of the  $\zeta$ -carbon of octanoate in acetoacetate.

#### DISCUSSION

According to the theory proposed in Paper I (1) that the  $\zeta$ - and  $\omega$ -carbons of octanoic acid give rise to a different species of 2-carbon fragment than that derived from carbons 1 to 6,  $\zeta$ -labeled octanoic acid should give an acetoacetate labeled predominantly in the carbonyl position.

Qualitative support for this hypothesis is provided by Experiments 3, 4, and 5 (Table I) in which  $\zeta$ -labeled octanoate was shown to give rise to acetoacetate containing on the average 3.3 times as much labeled carbon in the carbonyl as in the carboxyl position.

The magnitude of this distribution may be shown to account almost quantitatively for the asymmetrical distribution of isotope observed in the acetoacetate formed from carboxyl and  $\beta$ -labeled octanoate if the assumption is made that the 2-carbon fragment derived from the  $\delta$ - and  $\epsilon$ -carbons of octanoate ( $\delta$  fragment) is identical with the carboxyl and  $\beta$  fragments (arising from the carboxyl,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -carbons of octanoate). According to this assumption, the first 6 carbons of carboxyl-labeled octanoic acid will give rise to three identical 2-carbon units (carboxyl,  $\beta$ , and  $\delta$  fragments) containing a labeled carbon with one-third the isotope concentration of the original labeled carboxyl group, and the  $\zeta$ - and  $\omega$ -carbons will form a 2-carbon unit (terminal fragment) which differs from the first three and, therefore, does not contain labeled carbon. 1 mole of

<sup>1</sup> To be used henceforth to designate the ratio of the concentration of labeled carbon in the carbonyl position to that in the carboxyl position of acetoacetate.

carboxyl-labeled octanoic acid will thus give rise to 1 mole of unlabeled terminal fragments of which 0.77 mole will enter the acetyl portion of acetoacetate and 0.23 mole will enter the carboxyl portion of acetoacetate (according to the  $\overset{*}{\text{CO}}:\overset{*}{\text{COOH}}$  ratio of 3.3 observed in the acetoacetate derived from  $\zeta$ -labeled octanoic acid). In the complete conversion of 1 mole of carboxyl-labeled octanoate into 2 moles of acetoacetate, the 3 moles of labeled fragments will therefore be unequally distributed between the carbonyl and carboxyl positions; 1.23 moles must enter the carbonyl portion of the molecule and the remaining 1.77 moles will enter the carboxyl portion. The distribution of isotope between the carbonyl and carboxyl carbons for the acetoacetate formed from carboxyl-labeled octanoate may be calculated therefore as  $1.23/1.77 = 0.70$ . The observed value of 0.75 is in good agreement with this calculated value and, therefore, permits the assumption that the  $\delta$  fragment derived from octanoate is identical with the  $\beta$  and carboxyl fragments.

Although experiments in Paper I (1) clearly show that carbons 1 to 4 of octanoate are completely converted into 2-carbon fragments in the process of forming acetoacetate, the possibility that carbonyl-labeled acetoacetate arises from  $\zeta$ -labeled octanoate by a mechanism involving the direct conversion of carbons 5, 6, 7, and 8 to acetoacetate *without* the formation of 2-carbon fragments remains to be considered. This is ruled out, however, by the fact that both  $\beta$ - and carboxyl-labeled octanoate give rise to acetoacetate with essentially the same asymmetrical distribution of isotope. If only the first 4 carbons of octanoate give rise to 2-carbon fragments, and these are similar, then symmetrically labeled acetoacetate should arise by random condensation of these fragments from both  $\beta$ - and carboxyl-labeled octanoate. The failure to obtain symmetrically labeled acetoacetate from carboxyl and  $\beta$ -labeled octanoate, therefore, makes it necessary to postulate the formation of a chemically different 2-carbon fragment from the remaining 4 carbons of the chain. This, therefore, eliminates the possibility of the direct conversion of most if not all of carbons 5, 6, 7, and 8 to acetoacetate as an unbroken unit.

Diagram 2 shows the salient features of acetoacetate formation from carboxyl- and  $\zeta$ -labeled octanoate.

It will be noted that the carboxyl,  $\beta$ , and  $\delta$  fragments have been pictured with methylene groups in contrast to the terminal fragment which has been presumed to bear a methyl group. The terminal fragment differs from the other three in that its  $\alpha$ -carbon (a) does not arise by cleavage and (b) originally bears 3 hydrogen atoms. The tendency of this fragment to acetylate rather than to be acetylated suggests strongly that it differs structurally from the other three in the number of hydrogens on the



$\alpha$ -carbon atom rather than in the possession of a different functional group on carbon 1.

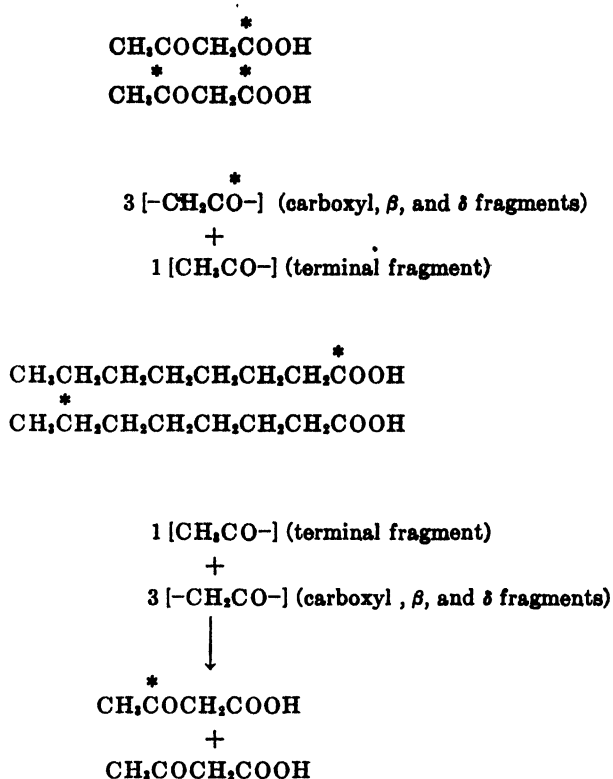


DIAGRAM 2. The conversion of octanoate to acetoacetate by a mechanism involving the *non-random* condensation of *two species* of 2-carbon intermediates.

\* Denotes the presence of labeled carbon.

In the interest of simplicity the relatively small incorporation of the  $\zeta$ -carbon of octanoate into the carboxyl position of acetoacetate has been omitted from Diagram 2. Its presence in the carboxyl position of acetoacetate may indicate that the terminal fragments are converted into fragments of the carboxyl,  $\beta$ , and  $\delta$  type at a rate which is relatively slow compared with the rate of direct utilization of intact terminal fragments in acetoacetate formation.

Experiments 6, 7, and 8 (Table I) show that the simultaneous conversion of unlabeled pyruvate to acetoacetate does not alter the distribution of the  $\zeta$ -carbon of octanoate in the final acetoacetate. This in turn indicates that the previously reported effect of unlabeled pyruvate in increasing

the  $\text{CO}^*:\text{COOH}^*$  ratio from 0.63 to 0.86 when simultaneously incubated with carboxyl or  $\beta$ -labeled octanoate (1) is not due to a mechanism effecting a more symmetrical distribution of the carbon of the terminal fragment of octanoate in acetoacetate. It may also be concluded that pyruvate does not give rise to a 2-carbon unit identical with this terminal fragment, since, if this were the case, the presence of non-labeled pyruvate should reduce the  $\text{CO}^*:\text{COOH}^*$  ratio in the acetoacetate formed from carboxyl- or  $\beta$ -labeled octanoate. If the assumption is made that pyruvate gives rise to 2-carbon units which are identical with the carboxyl,  $\beta$ , and  $\delta$  fragments of octanoate, the effect of non-labeled pyruvate in partially equalizing this ratio when incubated with carboxyl- or  $\beta$ -labeled octanoate is readily explained. If pyruvate is being oxidized at a rate sufficient to double the relative number of these fragments in existence at any moment during the oxidation of octanoate, it may be calculated<sup>2</sup> that the  $\text{CO}^*:\text{COOH}^*$  ratio should increase from 0.70 to 0.86. Similarly if pyruvate triples the relative number of these fragments, the ratio should increase from 0.70 to 0.91. In the experiments previously reported (1) the concentrations of pyruvate and carboxyl- or  $\beta$ -labeled octanoate were adjusted so that the rates of pyruvate and octanoate oxidation (and presumably the rate of 2-carbon unit production) were approximately equal.

Typical increases in the  $\text{CO}^*:\text{COOH}^*$  ratio found in such experiments in which the comparison was made on different portions of the same homogenate were 0.69 to 0.82 and 0.56 to 0.81 (1). It is possible therefore to account for this effect of pyruvate by assuming that it merely increases the relative number of fragments of the carboxyl,  $\beta$ , and  $\delta$  type.

Further evidence that pyruvate gives rise to a 2-carbon unit identical with the carboxyl,  $\beta$ , and  $\delta$  fragments of octanoate is found in experiments

<sup>2</sup> 1 mole of carboxyl-labeled octanoate will form 1 mole of unlabeled terminal 2-carbon fragments and 3 moles of labeled 2-carbon fragments (since the first three fragments are identical). If, simultaneously, 4 moles of pyruvate give rise to 4 moles of 2-carbon fragments which are identical with the labeled fragments from octanoate, the pyruvate fragments will mix with the labeled fragments to give 7 moles of labeled fragments now containing labeled carbons with one-seventh of the original isotope concentration in the carboxyl group of the octanoate. Since 0.77 mole of the *terminal* (non-labeled) fragment enters the carbonyl position of acetoacetate and 0.23 the carboxyl position, 3.23 moles ( $4.00 - 0.77$ ) of the *labeled* fragments must enter the carbonyl position and 3.77 ( $4.00 - 0.23$ ) the carboxyl position in order to complete the formation of the 4 moles of acetoacetate which 1 mole of octanoate, together with 4 moles of pyruvate, is capable of forming. The distribution of labeled carbon between the carbonyl and carboxyl positions in the acetoacetate then will be  $3.23/3.76 = 0.86$ .

(1) suggesting that pyruvate gives rise to a 2-carbon unit which is identical with "active" acetate and that "active" acetate in turn is identical with the carboxyl and  $\beta$  fragments of octanoate.

Considered together, all of the results reported in this study are most readily explained by the assumption that pyruvate, acetate, and the first 6 carbons of octanoate give rise to a common 2-carbon intermediate. This assumption does not appear to be in accord with the evidence of Bloch and Rittenberg (6), confirmed by Anker (7), that pyruvate unlike acetate is relatively incapable of acetylating aromatic amines or contributing to cholesterol formation *in vivo* when laboratory rats of a Wistar strain are used. Anker found very little difference between pyruvate and acetate in these respects when Sprague-Dawley rats were used (7). Our rats, however, of a Wistar strain, are to be compared with Anker's laboratory rats (also of a Wistar strain); therefore our data concerning pyruvate and acetate appear to be in conflict with the results obtained in the study of the acetylation of foreign amines.

It should be pointed out, however, that acetoacetate formation and the acetylation of foreign amines are not only different reactions but apparently occur in different phases of the liver cell. Lipmann has shown that the enzyme system responsible for the acetylation of sulfanilamide by acetate in pigeon liver (and presumably of other aromatic amines) is water-soluble (8), whereas the formation of acetoacetate from pyruvate and fatty acids has been shown by Lehninger to be associated with the insoluble residues of liver cells (9). Since, in our view, pyruvate and acetate appear to produce a common 2-carbon intermediate, it would follow that the transfer of acetyl carbon from pyruvate to aromatic amines should occur in the intact animal. However, the conversion of labeled pyruvate to this active 2-carbon unit by the insoluble particles within the liver cell would entail dilution of labeled carbon by endogenous 2-carbon units produced from fatty acids at this localized site so that the labeled carbon of pyruvate could be subject to greater dilution than that of acetate (which can be directly activated and utilized by the water-soluble acetylating enzyme system) in the process of providing acetyl groups for an aromatic amine.

In the light of these considerations and of the general possibility that intracellular structures may complicate the interpretation of isotope experiments on intact tissues and animals, we do not feel that the differences in isotope dilution observed in these acetylation studies *in vivo* rule out the possibility that pyruvate and acetate (and fatty acids) give rise to a common 2-carbon intermediate.

Further experiments will be necessary to explain why acetate and pyruvate, which both contain a methyl group, give rise to a 2-carbon fragment differing from the terminal fragment derived from fatty acids.

We wish to express our appreciation to Dr. D. Wright Wilson for his interest and advice.

#### SUMMARY

The synthesis of  $\zeta$ -(C<sub>7</sub>)-labeled octanoic acid is described.

The incubation of  $\zeta$ -labeled octanoate with the washed homogenate of rat liver resulted in the formation of predominantly carbonyl-labeled acetoacetate.

Simultaneous incubation of non-labeled pyruvate with  $\zeta$ -labeled octanoate did not alter the distribution of isotope in the resulting acetoacetate.

These experiments, along with previously reported studies with carboxyl- and  $\beta$ -labeled octanoate, demonstrate that fatty acids upon oxidation by liver give rise to two species of 2-carbon units in the process of forming acetoacetate. One species which is not readily acetylated is derived from the  $\zeta$ - and  $\omega$ -carbons of octanoate, while the other species arises from the first 6 carbons of octanoate.

The possibility that pyruvate and acetate form 2-carbon units which are identical with the 2-carbon units derived from the first 6 carbons of octanoate is discussed.

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# THE HYDROLYSIS OF NUCLEOPROTEINS BY CATHEPSINS FROM CALF THYMUS

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A combination of enzymatic and staining cytological techniques with ultraviolet spectroscopy has suggested the possible participation of nucleoproteins in the synthetic processes of cells (1-4). Little is known concerning the function of enzymes in the intracellular metabolism of nucleoproteins. One approach to the problem has been through the study of the action of enzymes upon nucleoproteins. Mazia, Hayashi, and Yudowitch (5) reported that the separated intracellular proteinases from liver did not hydrolyze the chromosomal proteins of the salivary glands of *Drosophila melanogaster*, but did hydrolyze the nucleoprotein substrate, chromosin, from calf thymus. Cohen (6) found that several extracellular proteolytic enzymes could hydrolyze the protein in thymus nucleohistone. The hydrolysis of two nucleoprotein preparations of thymus tissue by cathepsin preparations separated from the same tissue is described here.

## Materials and Methods

*Preparation of Nucleoprotein Fractions*—The two nucleoprotein fractions used as substrates were prepared as follows. 1200 gm. of cleaned and frozen calf thymus tissue were extracted in a blender with 5 liters of cold 0.325 M KCl. The suspension was filtered through fine gauze and centrifuged for 30 minutes at 2000 R.P.M. The pH was 6.4. The sediment which was designated NPI was very viscous and appeared microscopically as a dense mass of nuclei. The NPI was washed twice in the centrifuge with cold 0.325 M KCl and once with water and lyophilized. Analysis of four preparations of NPI showed an average of 46 per cent deoxyribonucleic acid (DNA), and only a trace of ribonucleic acid. The analyses for nucleic acids were made by extraction with hot trichloroacetic acid according to Schneider (7) and application of the colorimetric techniques of Dische (8) and Meibbaum (9).

The second nucleoprotein preparation was made from the NPI nucleoprotein by dissolving it in the minimal amount of 0.5 N NaOH with mechanical stirring in the cold. Solid ammonium sulfate was added to 30 per cent saturation (21 gm. per 100 ml.). The large globules of jellied protein which collected on the surface were filtered, washed with 21 per

cent ammonium sulfate, and dialyzed against cold running tap water. The precipitated protein was lyophilized. This preparation was designated NP30, and the content of DNA of three preparations was 58.3, 58.6, and 59.0 per cent. Although its use as a substrate is not reported here, a third protein preparation was obtained by saturating the filtrate from the NP30 with ammonium sulfate. The comparatively small amount of precipitate was dialyzed and lyophilized. This fraction contained very little phosphorus or DNA.

The method for the preparation of the calf thymus cathepsin preparations has been described previously (10, 11). Three of these preparations were used.

*pH-Activity Curves*—Digests were made containing 2.5 gm. of NPI or NP30 nucleoprotein in 100 ml. of citrate or phosphate buffers. These suspensions were emulsified in a blender and were slightly viscous. Portions of these substrate suspensions were adjusted electrometrically with 0.5 N alkali or acid to the required pH. To each 2.5 ml. of substrate 1 mg. of cathepsin preparation was added in the form of a dry powder. These digests and control suspensions of substrate were digested for 90 minutes at 39° in a water bath in which they were constantly agitated. The extent of the hydrolysis of the nucleoproteins was determined by measuring the increase in tyrosine color equivalent (12) or the increase in soluble nitrogen when the digests were precipitated with 0.3 M trichloroacetic acid.

*Determination of Catheptic Hydrolysis*—The degree of catheptic hydrolysis was determined by measuring the increase in soluble nitrogen and phosphorus in the digests. Since the soluble nitrogen and phosphorus represented, for the most part, the products of the hydrolysis which were dialyzable, the degree of hydrolysis could also be found by comparing the total nitrogen and phosphorus of the non-dialyzable products of hydrolysis with the total nitrogen and phosphorus of the original digests. Digests were made with 0.5 gm. of nucleoprotein and 20 mg. of thymus cathepsin preparation in 50 ml. of citrate buffer, with or without 50 mg. of cysteine hydrochloride. Control substrates without enzyme and with or without 1 mg. of cysteine hydrochloride per ml. were made. The digests and controls were incubated for 24 hours at 39° and then dialyzed for 24 hours against cold running tap water. The total nitrogen and total phosphorus of the digests and controls were determined before hydrolysis and after hydrolysis and dialysis. The soluble phosphorus was determined by sulfuric acid digestion of the supernatant obtained when the digests were precipitated with 0.3 M trichloroacetic acid, with use of the colorimetric technique of Fiske and Subbarow (13).

*Analysis of Non-Dialyzable Products of Hydrolysis*—In order to determine whether chemical changes had occurred in the nucleoproteins by

hydrolysis, the non-dialyzable material resulting from the catheptic digestion was lyophilized and analyzed for total nitrogen, total phosphorus, DNA, and for arginine by the method of Brand and Kassell (14). The values obtained were compared with the results of similar analyses of the original nucleoprotein preparations before hydrolysis.

*Analysis of Dialysates of Hydrolyzed Nucleoproteins for DNA*—In order to determine whether the dialyzable nitrogen and phosphorus of the digests represented nucleic acid which had been depolymerized after hydrolysis from the nucleoprotein, the dialysates were analyzed for DNA. Digests were made containing 1 gm. of NP30 in 50 ml. of citrate buffer, pH 5.75, and were incubated for 3 hours with 50 mg. of thymus cathepsin prepara-

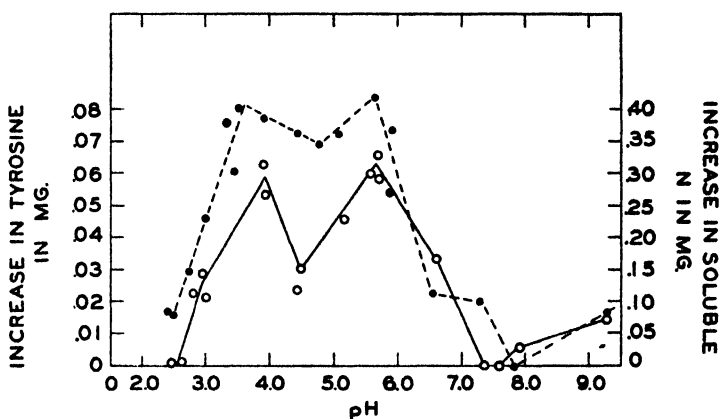


FIG. 1. The hydrolysis of thymus nucleoprotein NPI (46 per cent DNA) by a thymus cathepsin preparation. The digests contained 1 mg. of cathepsin and 62.5 mg. of nucleoprotein in 2.5 ml. of citrate or phosphate buffer and were digested 90 minutes at 37°. Increase in tyrosine, dash line; increase in soluble N, solid line.

tion. The same volumes of substrate without cathepsin were incubated as controls. Controls and digests were then dialyzed for 24 hours against 200 ml. of distilled water in the cold. The DNA in the dialysates was determined by ultraviolet light absorption at 2600 Å in a Beckman spectrophotometer with a known solution of DNA as a standard. The Dische test for DNA and the analysis of total organic phosphorus were made on these dialysates.

#### RESULTS AND DISCUSSIONS

Two pH optima in the acid range were found in the hydrolysis of the nucleoprotein, NPI, which contained 46 per cent DNA (Fig. 1). The second substrate used, NP30, which was derived from the NPI and contained 58 per cent DNA, showed three pH optima (Fig. 2). The same pH

optima were found for three different NPI preparations and two different NP30 preparations when hydrolyzed by three different cathepsin preparations. These optimal reactions in the acid range were characteristic of catheptic proteinase activity which usually occurs on the acid side of the isoelectric point of the protein.

The catheptic hydrolysis of NPI, as demonstrated by an increase in soluble nitrogen and phosphorus, was greater at pH 4.0 than at pH 5.8, both in the presence and absence of added cathepsin. There was less hydrolysis and subsequent loss by dialysis of nitrogen and phosphorus in control substrates which had been dialyzed without a period of incubation. The question arose, therefore, as to whether the autolysis of the substrates could be accounted for by the presence of small amounts of

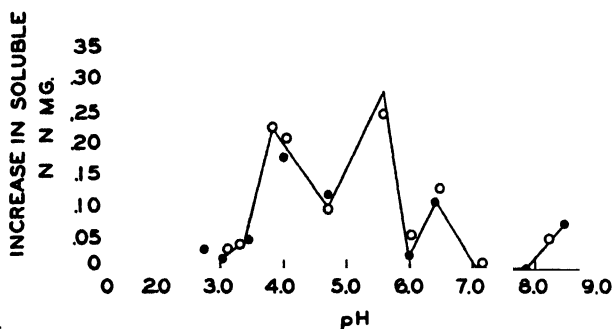


FIG. 2. The hydrolysis of thymus nucleoprotein NP30 (58 per cent DNA) by a thymus cathepsin preparation. The digests contained 1 mg. of cathepsin preparation and 62.5 mg. of NP30 nucleoprotein in 2.5 ml. of citrate or phosphate buffer and were digested 90 minutes at 37°.

cathepsin in the NPI preparations. When tested for catheptic activity, 10 mg. of an NPI preparation liberated 0.068 mg. of tyrosine color equivalent from 2.5 ml. of 2.5 per cent hemoglobin in 90 minutes. This degree of catheptic activity was equal to that shown by 1 mg. of a thymus cathepsin preparation acting for 90 minutes. The close association of cathepsin with this nucleoprotein fraction is of considerable interest, but the results of its use as a substrate could not be easily interpreted. Since the NPI fraction was derived largely from nuclei, its autolysis will be discussed in greater detail in a study of the catheptic activity of the separated nuclei of cells.

In the catheptic hydrolysis of the NP30 nucleoprotein about one-half of the nitrogen and phosphorus was made soluble and then lost by dialysis when cysteine was added at pH 5.2 (Table I). In the absence of added cathepsin there was a slight increase of soluble phosphorus and no loss of



phosphorus on dialysis alone. About 30 per cent of the nitrogen of the NP30 was dialyzable with and without a period of incubation. It appeared that the depolymerizing activity of the adherent cathepsin of NPI had been lost in the process of deriving the NP30 preparation from the NPI

TABLE I  
*Hydrolysis of Thymus Nucleoprotein NP30 by Thymus Cathepsin Preparation*

Composition of digests	Analysis of digest			
		Before hydrolysis	After hydrolysis	After hydrolysis and dialysis*
		per cent	per cent	per cent
Dialysis only	Total N	100		71.98
Nucleoprotein, NP30	" P	100		100.00
Citrate buffer, pH 5.2	Soluble N†	17.85		8.68
	" P†	0.25		0.81
Nucleoprotein, NP30	Total N	100		72.79
Citrate buffer, pH 5.2	" P	100		100.00
	Soluble N	16.76	19.41	7.31
	" P	2.0	2.21	0
Nucleoprotein, NP30	Total N	100		54.17
Cathepsin	" P	100		53.13
Citrate buffer, pH 5.2	Soluble N	17.57	34.30	13.00
	" P	2.10	40.63	10.81
Nucleoprotein, NP30	Total N	100		70.09
Citrate buffer, pH 5.2	" P	100		100.00
Cysteine, HCl	Soluble N	17.70	16.27	3.38
	" P	0	0	0
Nucleoprotein, NP30	Total N	100		46.07
Cathepsin	" P	100		50.09
Citrate buffer, pH 5.2	Soluble N	17.81	40.29	9.91
Cysteine, HCl	" P	0	52.21	1.61

\* The percentages of total nitrogen and phosphorus after dialysis were calculated by using the total nitrogen and phosphorus of the original digest as 100 per cent.

† The percentages of soluble nitrogen and phosphorus were calculated by using the total nitrogen and phosphorus as 100 per cent.

by solution in alkali, precipitation with ammonium sulfate, and dialysis. The NP30 preparations showed no catheptic activity on hemoglobin. There was a slight but definite increase in soluble nitrogen and phosphorus when cysteine was added to activate the cathepsin.

*Products of Catheptic Hydrolysis of Nucleoprotein*—Analysis of both the

non-dialyzable products of hydrolysis and the dialysates indicated that the nucleoprotein preparations had lost nucleic acid by the catheptic hydrolysis. As seen in Table II the DNA content (from the Dische desoxyribose reaction) of the NPI had decreased from 46.12 to 31.20 per

TABLE II

*Analysis of Thymus Nucleoprotein Preparations before Hydrolysis and of Non-Dialyzable Products of Hydrolysis after Lyophilization*

Content of digest	Analysis		
		Before hydrolysis	After hydrolysis, dialysis, and lyophilization
		<i>per cent</i>	<i>per cent</i>
Dialysis only	N	13.69	13.21
Nucleoprotein, NPI	P	2.93	3.17
Citrate buffer, pH 5.75	DNA	46.12	45.53
	Arginine	5.31	5.32
Nucleoprotein, NPI	N	13.69	13.40
Citrate buffer, pH 5.75	P	2.93	3.01
Cysteine, HCl	DNA	46.12	36.35
	Arginine	5.31	5.92
Nucleoprotein, NPI	N	13.69	14.19
Cathepsin	P	2.93	2.61
Citrate buffer, pH 5.75	DNA	46.12	31.20
Cysteine, HCl	Arginine	5.31	6.87
Dialysis only	N	15.95	13.36
Nucleoprotein, NP30	P	3.95	3.79
Citrate buffer, pH 5.2	DNA	58.32	59.61
	Arginine	3.42	4.02
Nucleoprotein, NP30	N	15.95	13.32
Cysteine, HCl	P	3.95	3.65
Citrate buffer, pH 5.2	DNA	58.32	54.22
	Arginine	3.42	3.85
Nucleoprotein, NP30	N	15.95	14.75
Cathepsin	P	3.95	2.97
Citrate buffer, pH 5.2	DNA	58.32	33.51
Cysteine, HCl	Arginine	3.42	5.40

cent, and for the NP30 had decreased from 58.32 to 33.51 per cent after hydrolysis and dialysis. In the dialysates the quantities of phosphorus, desoxyribose, and purines and pyrimidines (by ultraviolet absorption) were found in the proportions present in DNA. As an example, one dialysate

of 222 ml. from 50 ml. of catheptic digest of NP30 contained 1.28 mg. of organic phosphorus, 14.82 mg. of DNA by the Dische colorimetric method, and 15.04 mg. of DNA by the ultraviolet absorption in the Beckman spectrophotometer. No inorganic phosphorus was found. Similar quantitative studies of the dialysates of many catheptic digests of preparations of NPI and NP30 confirmed these results.

It was evident from the loss of DNA into the dialysates that the cathepsin preparations had not only hydrolyzed the protein of the nucleoproteins but had depolymerized the liberated DNA, rendering it dialyzable. In this respect the cathepsins acted differently from the extracellular digestive enzymes used by Cohen (6) who found that trypsin, chymotrypsin, and ribonuclease liberated DNA from thymus histone as indicated by an increase in the viscosity of the digests. However, the DNA was not depolymerized by these enzymes and was not dialyzable. The depolymerizing properties of calf spleen and calf thymus preparations on DNA and ribonucleic acid are described in the accompanying paper (15).

The non-dialyzable products of the hydrolysis of both nucleoproteins showed a percentage increase in arginine (Table II). This increase amounted to about 30 per cent of the original arginine content with the NPI and 57 per cent with the NP30 preparation. This increased percentage of arginine in the non-dialyzable hydrolysis products might suggest that the part of the nucleoproteins containing arginine was less readily hydrolyzed by cathepsin.

Von Euler and Hahn (16) reported the possibility that a potent proteolytic enzyme system acted upon the nucleoproteins of the nucleus and prevented the isolation of nucleoproteins with the same antigenic properties as the intact nuclei. Since our nucleoproteins were extracted by blending in a weak KCl solution, many nuclei were probably broken and, therefore, no statement can be made from these experiments concerning the localization of cathepsins in the cell. At present the experimental procedure used to separate the cathepsins suggests that they come principally from the cytoplasm. Studies on the catheptic activities of particulate fractions of the cell are under way in this laboratory.

#### SUMMARY

Two nucleoprotein preparations from calf thymus, one with 46 per cent and one with 58 per cent desoxyribonucleic acid, were hydrolyzed by calf thymus cathepsin preparations. pH optima in the acid range were obtained for this hydrolysis. Analysis of the non-dialyzable products of the catheptic hydrolysis of the nucleoproteins as well as of the dialysates showed that the nucleic acid had been hydrolyzed from the nucleoproteins and depolymerized, thus rendering it dialyzable. There was an increase in

the percentage of arginine in the nucleoprotein residues after hydrolysis and dialysis.

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# THE NUCLEASE ACTIVITIES OF CATHEPSIN PREPARATIONS FROM CALF SPLEEN AND THYMUS

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In a study of the rôle of cathepsins in nuclear metabolism (1) these intracellular proteinases were found to hydrolyze thymus nucleoproteins and render dialyzable the desoxyribonucleic acid. Further characterization of this nuclease activity of the cathepsin preparations revealed pH optima in the acid range for both desoxyribonucleic acid (DNA) and ribonucleic acid (RNA). This was in contrast to the specific activities of the pancreatic nucleases which show pH optima above neutrality (2-4). Evidence which establishes an intracellular desoxyribonuclease and ribonuclease activity distinct from that of the pancreas and a correlation of the factors which affect the proteolytic and nuclease activities of the cathepsin preparations are reported here.

## *Materials and Methods*

*Preparation of Enzymes*—The method used for the separation of the cathepsin preparations from frozen calf spleen and thymus tissue has been described (5, 6). The extracts of the tissues were made by grinding the frozen tissue in a mortar and extracting with 4 volumes of water. The suspension was filtered through gauze and centrifuged. The supernatant was used after dilution with an equal quantity of buffer of the required pH.

*Determination of Enzymatic Activities*—The desoxyribonuclease activities were determined by measuring the rate of decrease of the relative viscosity of solutions of DNA in Ostwald-Fenske type viscosimeters in a water bath at 39°. In these determinations 1 ml. of extract or 1 ml. of water containing 1 mg. or less of cathepsin preparation was added to 5 ml. of a buffered solution containing 10 mg. of DNA. After the viscosimetric determinations were made, the digestion was continued for 1 hour and aliquot samples were removed for the determination of soluble DNA or soluble phosphorus. The insoluble DNA was precipitated by the addition of an equal volume of 20 per cent trichloroacetic acid. After centrifugation the soluble DNA was determined by the method of Dische (7) and the soluble phosphorus by the method of Fiske and Subbarow (8).

The ribonuclease activity was measured by the increase in soluble phosphorus after digestion of RNA by the extracts of the tissues or by

the cathepsin preparations. To 1 ml. of a buffered solution containing 10 mg. of the sodium salt of RNA was added 1 ml. of a 1:8 tissue extract or 1 ml. of a buffered solution of 1 mg. of cathepsin preparation. After digesting 1 hour at 39°, 2 ml. of uranylacetate-trichloroacetic acid solution (MacFadyen (9)) were added to precipitate the insoluble RNA. After centrifugation the total soluble phosphorus in the supernatant was determined as described above for the DNA experiments. The soluble phosphorus was determined in control digestion mixtures in which water replaced the tissue extract or cathepsin solution and in which water replaced the RNA solution.

The catheptic activities were determined by a modification of Anson's technique (10).

Cysteine hydrochloride was added to the enzyme solutions before digestion, with use of 1 mg. per mg. of cathepsin preparation. The *p*-chloromercuribenzoate, which was used as an inhibiting agent, was prepared according to the method of Whitmore and Woodward (11).

*Preparation of Substrates*—The sodium salt of desoxyribonucleic acid was prepared according to Hammarsten (12). The sodium salt of yeast nucleic acid was prepared by dissolving commercial yeast nucleic acid in 0.5 N NaOH at 5° and precipitating with 10 volumes of glacial acetic acid. The precipitate was washed with water in the centrifuge tubes and dried with alcohol and ether. The dried nucleic acid was again dissolved in 0.5 N NaOH in the cold and dialyzed against cold running tap water. The sodium nucleate was precipitated by the addition of 8 volumes of alcohol and the minimum amount of solid sodium acetate needed to obtain complete precipitation. The final product was dried with alcohol and ether.

### Results

*pH Activity Curves*—The rate of the progressive decrease in the relative viscosity of the DNA solutions containing the cathepsin preparations was constant when the equation for a first order reaction was applied. Since the amount of protein added as enzyme was relatively very small, the viscosity reading immediately after this addition was used to calculate the relative viscosity at zero time. As pointed out by Laskowski and Seidel (4), with the use of this method it was assumed that at any given time of digestion the relative viscosity of the reacting mixture was proportional to the concentration of the unattacked DNA. Under the conditions of these experiments the error involved was small. The *K* values for the desoxyribonuclease activity of the spleen and thymus cathepsin preparations were highest at pH 4.5 (Figs. 1 and 2). The viscosity of solutions of DNA decreases markedly below pH 4.0 and above pH 7.5, as shown by Vilbrandt and Tennent (13). The *K* values for the rate of decrease of

viscosity could, therefore, not be obtained in the more acid range to complete the curve. However, the pH activity curve based upon the soluble DNA liberated by the cathepsins also showed an optimum at pH 4.5 and this curve could be completed. The comparative nuclease activities of the enzyme preparations from spleen and thymus corresponded to their respective catheptic activities on hemoglobin in which the spleen cathepsin liberated 1.25 mg. of tyrosine color equivalent and the thymus cathepsin 0.68 mg. (14).

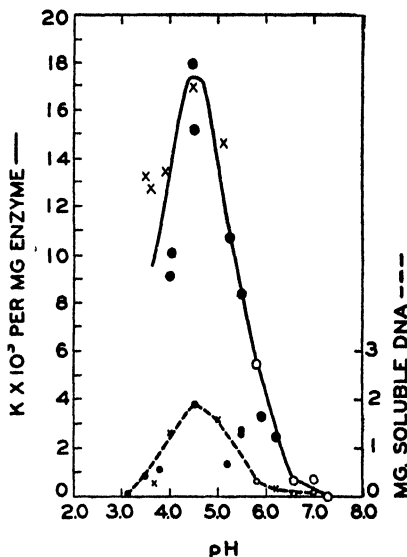


FIG. 1

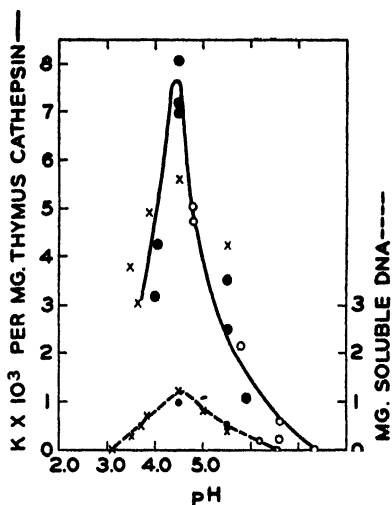


FIG. 2

FIG. 1. Desoxyribonuclease activity of spleen cathepsin preparation.  $K$  values obtained for rate of decrease in relative viscosity. The buffers used were citrate X, acetate ●, and veronal ○.

FIG. 2. Desoxyribonuclease activity of thymus cathepsin preparation.  $K$  values obtained for rate of decrease in relative viscosity. The buffers used were citrate X, acetate ●, and veronal ○.

In order to determine whether nucleases of the pancreatic type were present in other tissues, and since earlier studies by Greenstein (15, 17) had reported the nuclease activities of tissue extracts at or near neutrality, the pH activity curves of extracts of spleen and thymus tissue were studied. With DNA as substrate these extracts also showed a pH optimum at pH 4.5 when the decrease in relative viscosity and the increase in soluble phosphorus were determined (Figs. 3 and 4). The catheptic activities of these extracts per mg. of nitrogen were equal, but the spleen extracts were about 2 to 3 times as active per ml. of extract. Other water-soluble

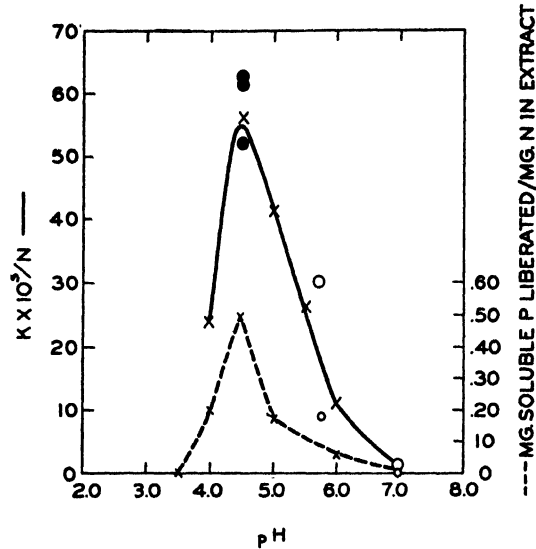


FIG. 3. Desoxyribonuclease activity of spleen extract.  $K$  values obtained for rate of decrease in relative viscosity. The buffers used were citrate X, acetate ●, and veronal O.

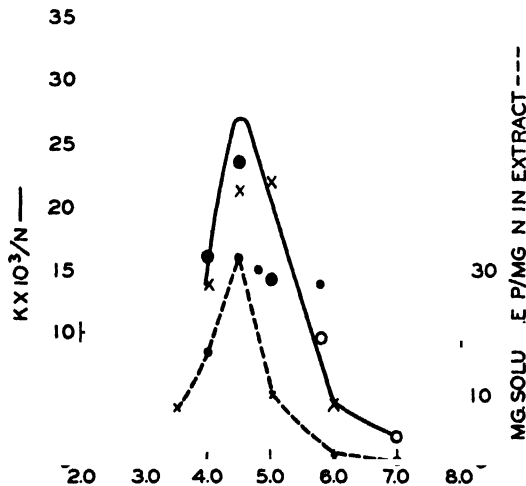


FIG. 4. Desoxyribonuclease activity of thymus extract.  $K$  values obtained for rate of decrease in relative viscosity. The buffers used were citrate X, acetate ●, and veronal O.

tissue proteins such as the albumins which did not have catheptic activity did not lower the viscosity of solutions of DNA under the conditions of these experiments.



**Ribonuclease Activity**—The ribonuclease of the thymus cathepsin preparations was most active at pH 5.0; that of the spleen preparations was most active at pH 5.5 (Fig. 5). Both preparations showed more activity above pH 6.0 on RNA than on DNA. The wider range of pH values at which the RNA was attacked may be due to its more heterogeneous composition. The ribonuclease of the spleen extracts was most active at pH 4.5 with citrate buffer, but with veronal buffer the range of high activity was broad, with considerable activity up to pH 6.5. The thymus extracts showed a definite pH optimum at pH 4.5 and a smaller peak of activity at pH 6.0 (Fig. 6).

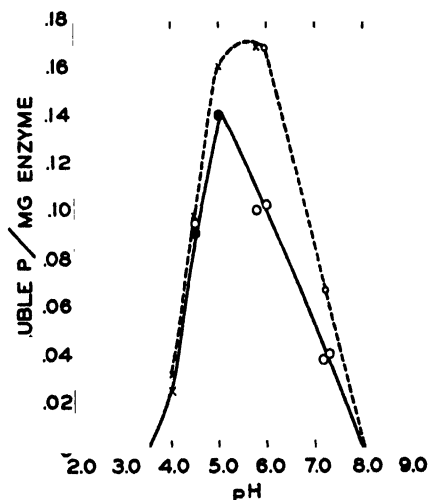


FIG. 5. Ribonuclease activities of spleen (dash line) and thymus (solid line) cathepsin preparations as measured by an increase in soluble phosphorus. The buffers used were citrate X, acetate ●, and veronal ○.

**Effect of Cysteine upon Nuclease Activities of Cathepsin Preparations**—The rate of decrease in relative viscosity of DNA solutions was accelerated when cysteine hydrochloride was added to the solutions of the cathepsin preparations. The increased rate was not constant, but, as seen in Table I, it was greater than could be accounted for by the addition of cysteine alone. The increase of the proteinase activities of cathepsin preparations by cysteine and cyanide has been demonstrated (5, 14, 18). Similar activation was found when determinations of the nuclease activity on DNA and RNA were followed by determinations of the increase in soluble phosphorus (Table II).

There was no evidence of the liberation of inorganic phosphorus by the cathepsin preparations either from DNA or RNA.

*Inhibition of Nuclease Activities*—The addition of magnesium sulfate produced a marked inhibitory effect upon the desoxyribonuclease activity of the cathepsin preparations (Table II). This was in marked contrast to the activation produced by magnesium sulfate on the pancreatic desoxyribonuclease (3, 4). Since the proteinase activities of these cathepsin preparations were not affected by the addition of magnesium sulfate, it is possible that the inhibition was due to a combination of the magnesium sulfate with the DNA.

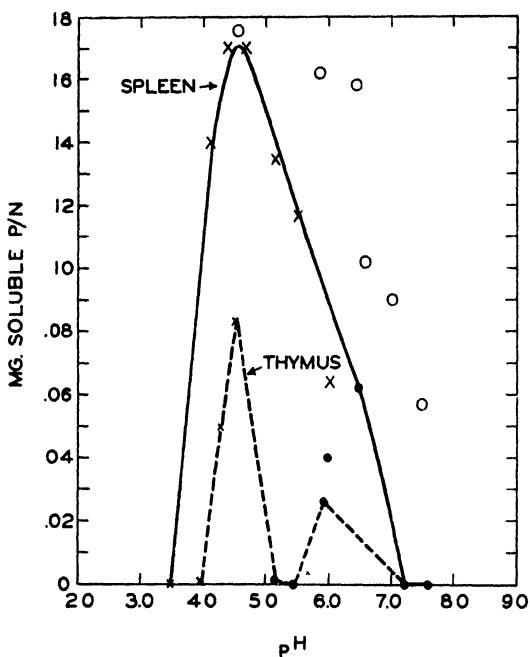


FIG. 6. Ribonuclease activities of spleen and thymus extracts, as measured by an increase in soluble phosphorus. The buffers used were citrate X, acetate ●, and veronal O.

The sulfhydryl-testing reagent, sodium *p*-chloromercuribenzoate, inhibited the nuclease activities of the cathepsin preparations (Table II). The sensitivity of the proteinase activity of the cathepsin preparations to this and other SH reagents has been reported (5, 6, 14, 18). The inhibitory effect upon DNA when measured by the increase in soluble phosphorus was much greater than when it was determined by the decrease in relative viscosity, suggesting that the points of attack by the enzyme on the highly polymerized DNA may be different. Further evidence of this phenomenon was seen when the DNA solutions of pH 3.5 had very low

TABLE I

*Effect of Cysteine on Desoxyribonuclease Activity of Spleen Cathepsin Preparation*

Time intervals	pH	K per mg.*		
		Spleen cathepsin preparation	Spleen cathepsin + cysteine†	Cysteine†
min.				
5	4.5	0.01569	0.02306	0.001806
10			0.02604	
15		" 0.01683	0.01844	0.000799
20		0.01560	0.01668	
25		0.01302		
30				0.000706
50	6.2			0.000489
60				0.000483
10		0.002119		
14			0.005911	
20		0.002461		0.001101
24			0.005015	
30		0.002636		0.001430
34			0.005091	
44			0.004691	
50		0.002542		0.000761

\* The  $K$  values were obtained by using the equation for a first order reaction,  $K = 1/t \times \log N_0/N_t$ , in which  $N_0$  was the relative viscosity at zero time and  $N_t$  at the time of incubation in minutes.

† 1 mg. of cysteine hydrochloride was added per mg. of cathepsin preparation.

TABLE II

*Activation and Inhibition of Nuclease Activities of Cathepsins*

Source of cathepsin	pH	Activation with cysteine		Inhibition with <i>p</i> -chloromercuribenzoate*			Inhibition with $MgSO_4$			
		DNA	RNA	DNA		RNA	0.01 M DNA		0.03 M DNA	
		Soluble P	Soluble P	Soluble P	Viscosity	Soluble P	Soluble P	Viscosity	Soluble P	Viscosity
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Spleen	4.5	40	45	89	55	27	39	63	74	96
	7.12	120	130							
Thymus	4.5	25	24	100	32	23	40	55	68	95
	7.12	68	66							

\* 5  $\mu M$  per mg. of enzyme preparation.

relative viscosity but no increase in soluble phosphorus unless they had been acted upon by the cathepsins.

*Effect of Heating upon Catheptic and Nuclease Activities of Cathepsin Preparations*—When heated in buffered solutions at pH 4.5, the cathepsin preparations lost their catheptic activity more rapidly than their nuclease activities. Fig. 7 shows the effect of heating a thymus cathepsin preparation. On heating 10 minutes at 70° in a water bath all of the catheptic activity was destroyed, while 50 per cent of the desoxyribonuclease and 27 per cent of the ribonuclease remained. All of the activities were destroyed by heating at 80° for 10 minutes at pH 4.5.

It is possible, of course, that heating under different conditions may provide a means for separating the proteolytic and ribonuclease activities as Kleczkowski (19) and McDonald (20) have demonstrated for the crystal-

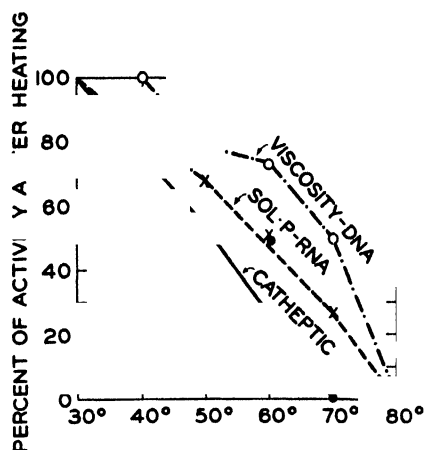


FIG. 7. The effect of heating on thymus cathepsin preparations at pH 4.5

line ribonuclease from the pancreas. There was no evidence that the intracellular ribonuclease was heat-stable, as has been shown for the corresponding enzyme from the pancreas.

#### DISCUSSION

Greenstein and Jenrette (15) and Greenstein (16, 17) found that the highly polymerized DNA and RNA were depolymerized by extracts of normal and neoplastic tissues at about pH 7.0. The pH-activity curves obtained in this study indicated that the optimal pH for the nuclease activities of spleen and thymus extracts and of the cathepsin preparations separated from these tissues was in the acid range. Catchside and Holmes (21) found that desoxyribonuclease preparations from spleen were most active at pH 5.0.

The intracellular desoxyribonuclease was differentiated from that of

the pancreas by the fact that concentrations of magnesium sulfate which activate the pancreatic desoxyribonuclease (3, 4) were inhibitory for the spleen and thymus desoxyribonucleases. There was further differentiation in the heat lability of the spleen and thymus ribonucleases as contrasted with the heat stability of the corresponding pancreatic ribonuclease.

The close correlation between the activating and inhibiting factors for the nuclease and proteolytic activities of these cathepsin preparations suggests either that they may be functions of a complex cathepsin molecule or that they are separate enzymes with very similar physical and chemical properties. The fact that cysteine acted as an activating and *p*-chloromercuribenzoate as an inhibiting agent would support the view that the nuclease as well as the proteinase function of the cathepsin preparations was dependent in part upon the SH groups in the enzyme-protein.

#### SUMMARY

Intracellular nucleases closely associated with the cathepsin preparations from thymus and spleen tissue were found to act optimally at pH 4.5 on DNA and at pH 5.0 to 5.5 on RNA. Extracts of these tissues also showed pH optima in the acid range.

The spleen cathepsin preparations used were correspondingly more active than the thymus preparations, both as proteinases and as nucleases.

The nuclease activities of the spleen and thymus cathepsin preparations were increased by cysteine and inhibited by *p*-chloromercuribenzoate, agents which respectively activate and inhibit the catheptic action of the preparations probably through SH groups on the enzyme-protein.

The intracellular nucleases associated with the cathepsins were differentiated from the pancreatic nucleases by the following: (1) there was optimal activity in the acid range, (2) the desoxyribonuclease was inhibited by magnesium sulfate in concentrations which activate the pancreatic desoxyribonuclease, (3) the ribonuclease was heat-labile, while the ribonuclease from the pancreas was heat-stable.

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# FURTHER STUDIES ON THE FACTOR REQUIRED BY *LEUCONOSTOC CITROVORUM* 8081\*

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Recently it was noted that *Leuconostoc citrovorum* 8081 failed to grow on a synthetic medium unless supplied with certain preparations from liver or other crude materials (1). Commercial preparations useful against pernicious anemia proved to be particularly active for this strain of *L. citrovorum*. The present report deals with preliminary attempts to concentrate the active principle and to differentiate it from other biologically active agents.

## Methods

The basal medium and method of assay for the active principle have been described previously (1). The results are expressed as "*citrovorum* units," defined as the amount of active material required per ml. of medium to permit half maximal production of acid by the organism. In the present study, most assays involved both turbidimetric measurement after 16 hours and measurement of acid production after 72 hours. The two measurements were desirable because moderately high amounts of folic acid have been observed to stimulate growth and acid production by *L. citrovorum* during a 72 hour interval (1), although these amounts were inactive during the first 36 hours. The two measurements were also useful in distinguishing between the activity due to thymidine and that due to the active principle in the liver concentrates.

*Differences between Liver Factor, Thymidine, and Folic Acid*—Thymidine has been observed to stimulate the growth of *L. citrovorum* 8081 (1) and of certain other organisms of the lactic group (2-4). Fig. 1 indicates the growth of *L. citrovorum* 8081 after 12, 18, and 28 hours in media containing graded amounts of liver concentrate (Reticulogen, Lilly) or of thymidine. The responses to the liver concentrate were regular and increased with concentration as well as with the time of incubation. The

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responses to thymidine, on the other hand, were only a fraction of those possible with the liver concentrate, with no consistent relationship between the amount of thymidine in the medium and the growth of the organism. The relative effect of the thymidine with respect to the liver concentrate increased as the period of incubation was prolonged, but even after 28 hours the maximal turbidity achieved with thymidine was much less than that observed in the presence of the liver concentrate; increased concentrations of thymidine failed to increase turbidity further. After 72 hours of incubation, acid production in the presence of thymidine was similar to that in the presence of a typical liver concentrate (Fig. 2), although the two curves were not identical.

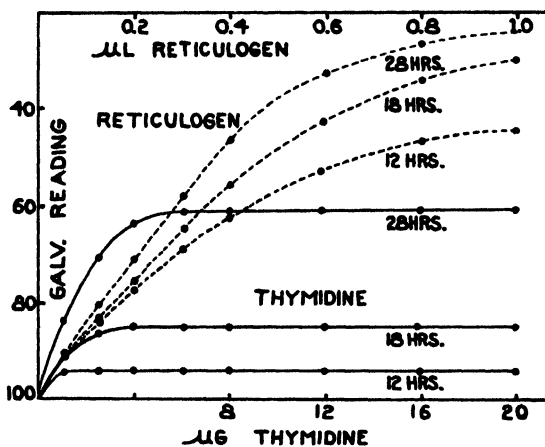


FIG. 1. Response of *L. citrovorum* to graded amounts of the liver concentrate Reticulogen and to thymidine. Turbidity measurements through Filter 660 of the Evelyn colorimeter with the uninoculated tube set at 100. All concentrations are amounts per 10 ml. of final medium. Broken lines, Reticulogen; solid lines, thymidine.

Folic acid, as reported previously (1), stimulated acid production by *L. citrovorum* after 72 hours of incubation, with, however, a distinct lag in the curve (Fig. 2); neither growth nor acid production was observed at concentrations of 0.8  $\gamma$  of folic acid per ml. of medium (1.6  $\gamma$  per tube), although increments of folic acid above this level resulted in regular increases in acid production by the organism. By way of contrast the amount of folic acid in 0.025 ml. of Reticulogen (the amount per ml. necessary for half maximal growth on a medium (1, 5) which itself contains 0.01  $\gamma$  of folic acid per ml.) is only 0.0002  $\gamma$ . It would therefore appear that folic acid is not the critical agent required by *L. citrovorum*, but rather that large amounts of folic acid enable the organism to produce a *citrovorum* factor (6).



Turbidity measurements after shorter periods of incubation likewise favor this conclusion. Previous results indicated that moderately high amounts of folic acid were inactive in stimulating the growth of *L. citrovorum* within a period of 36 hours or less (1). Very high amounts of folic acid, however, stimulate the initial growth of the organism. The concentration of folic acid needed for some growth to occur in 24 hours or less (as illustrated in Fig. 3) has not, however, been established with certainty; the minimal concentration needed to overcome the initial lag appears to depend among other things on the condition of the culture, and on the size of the inoculum.

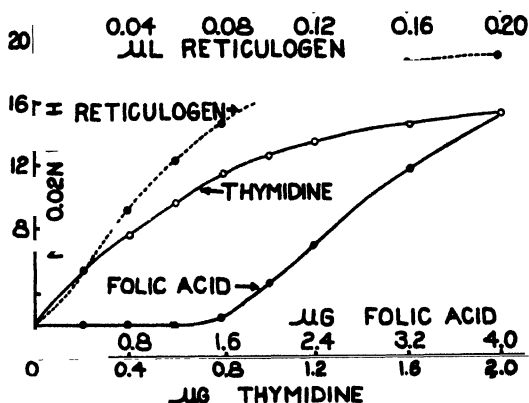


FIG. 2. Acid production by *L. citrovorum* after 72 hours in media containing thymidine, folic acid, or the liver concentrate Reticulogen. All concentrations are amounts per tube containing 2.0 ml. of final medium.

*Preliminary Concentration of Factor*—Lilly Reticulogen 360 (40,000 *citrovorum* units per ml.) was used as the starting material. 10 ml. of the liver concentrate were diluted with 10 ml. of water and adjusted to pH 3.0 with 5 N H<sub>2</sub>SO<sub>4</sub>. The precipitate which formed was removed by filtration. 1 gm. of norit was added to the filtrate and stirred with an electric stirrer for 20 minutes at room temperature. The mixture was then filtered and the norit washed by suspending it in 50 ml. of 50 per cent ethanol and stirring for 15 minutes at room temperature. The mixture was then filtered and the active fraction eluted from the norit by suspending it in 50 ml. of a mixture of ammonia, water, and ethyl alcohol (15 ml. of concentrated NH<sub>4</sub>OH + 25 ml. of ethyl alcohol + 10 ml. of water) at 70° with stirring for 30 minutes. The elution was repeated and the eluates combined and reduced to dryness by distillation under a vacuum (water pump, 20 mm. of Hg) with the aid of a water bath heated to 70°.

The 121 mg. of solids obtained, Concentrate A, were then dissolved in 50 ml. of water, the pH adjusted to 3.0 with  $H_2SO_4$ , the solution filtered, 0.25 gm. of norit added, and the procedure repeated as above. 27 mg. of solids, Concentrate B, were obtained which proved to be highly active in stimulating growth and acid production by *L. citrovorum*. The response of the organism to graded amounts of Concentrate B was qualitatively identical with its response to Reticulogen, both in the production of acid and in turbidity (Fig. 3). 1 mg. of Concentrate B contained 3840 *citrovorum* units; 1 unit, therefore, was supplied by only 0.26  $\gamma$  of the material. Similar values were obtained whether the analyses were based on acid production or on turbidity. For an equivalent production of acid 0.24  $\gamma$  of

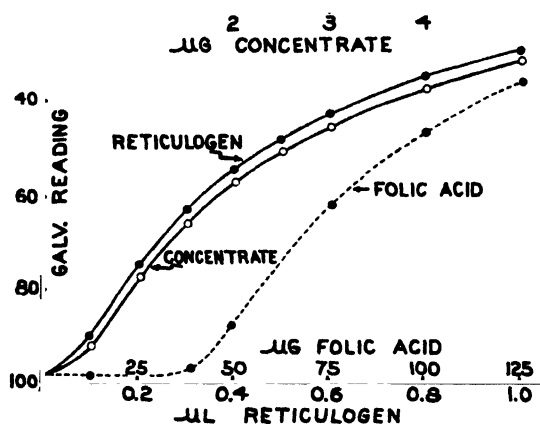


FIG. 3. Response of *L. citrovorum* to graded amounts of Reticulogen, folic acid, or of Concentrate B as determined by turbidity measurements after 16 hours with the Evelyn colorimeter (Filter 660 with an uninoculated tube set at 100 as the blank). All concentrations are amounts per 10 ml. of final medium.

thymidine was necessary (Fig. 2). Attempts to equate the turbidimetric activities of thymidine and of Concentrate B after 16 hours of incubation were impossible, since, even with relatively high amounts of thymidine in the medium, the turbidity failed to reach that corresponding to 1 *citrovorum* unit (Fig. 1).

**Other Concentrates**—A series of concentrates of the antipernicious anemia principle was tested with *L. citrovorum*, and their activities found to range from 4000 to 40,000 *citrovorum* units per ml. The dry weight ranged from 2.5 to 23 per cent. Preparation L-5 (1), containing 23 per cent of dry matter, was inactive for chicks, rats, or *Lactobacillus lactis* Dorner, but it was relatively active for *L. citrovorum*, 32,000 units per ml. 80 ml. of this solution were subjected to the concentration procedure described, yielding approximately 250 mg. of dry material, Concentrate C, the

*citrovorum* activity of which was 8330 units per mg. or 1 *citrovorum* unit in 0.12  $\gamma$ . This preparation, therefore, was about twice as active as the preparation prepared from Reticulogen, and it was at least twice as active as thymidine. Qualitatively, the standard curves obtained with Concentrate C were indistinguishable from those obtained with Reticulogen or with Concentrate B.

*Effect of Other Factors on L. citrovorum*—In spite of the qualitative similarity in the responses of *L. citrovorum* to Concentrates A, B, and C and to cruder liver preparations, the possibility remained that the organism required several unknown factors, all of which were supplied by the liver concentrates, and that the failure of the organism to respond completely to substances such as thymidine or folic acid was due to the absence from the medium of a second factor. Accordingly, oleic acid, streptogenin (enzymatic digest from casein (7)), tomato juice, or cobalt was added to the basal medium and the effects of the thymidine and folic acid as well as of the liver concentrates toward *L. citrovorum* were reexamined. None of these factors caused the growth of *L. citrovorum* on the synthetic medium or affected its response to the known growth factors or the liver concentrates.

*Relation of citrovorum Factor to Vitamin B<sub>12</sub> and to Principles Required by Rat and Chicken*—It was observed previously that certain preparations from fish solubles known to be active in stimulating the growth of chicks (8-11) were inactive for *L. citrovorum* (1), and that the relative activities of a series of preparations from liver were not the same for chicks or rats as for the microorganism. Since then it has been reported that vitamin B<sub>12</sub> stimulates the growth of chicks and rats and has many of the properties of the animal protein factor (12, 13). This would suggest that the factor stimulating the growth of *L. citrovorum* was not vitamin B<sub>12</sub>, and in support of this suggestion a lack of correlation between the clinical activities of a series of liver preparations and their activities for *L. citrovorum* (1) might be cited, although the latter evidence is not in itself conclusive in view of the variations inherent in the clinical assay.

Table I presents the biological activities of a series of liver preparations for *L. citrovorum* 8081, the anemia patient, the chick, the rat, and *L. lactis* Dorner. For the latter results we are indebted to Dr. W. E. Gaunt and Dr. S. A. Shannon of The Squibb Institute for Medical Research, under whose direction the analyses were made before the details of the method were generally available. Usually the responses of the rat and chick resembled those of *L. lactis* Dorner much more closely than those of *L. citrovorum* (Table I, Samples L-2, L-4, L-5, F-21, and especially L-16), and for most of these the correlation between the results of the clinical assays and those with *L. lactis* Dorner was also fairly high. Presumably, therefore, the active agent being measured by all of these species, rat,

chick, man, and *L. lactis* Dorner, was vitamin B<sub>12</sub>. On the other hand, Sample L-5 did not stimulate the growth of rats and chicks, and it was almost devoid of activity for *L. lactis* Dorner, although its activity was sufficiently high to warrant its use as the starting material for our relatively potent Concentrate C. Sample L-5 was reputed to be clinically active. Another exception to the general rule was Sample L-11; its clinical activity was 50 per cent that of Sample L-2, Reticulogen, and its activity for *L. citrovorum* was also high, while it was not particularly active for *L. lactis* Dorner. All other results, however, suggest that the *citrovorum* factor is distinct from vitamin B<sub>12</sub>, and this has been confirmed by direct determination; the addition of vitamin B<sub>12</sub> to the basal medium in amounts up to

TABLE I

*Comparative Biological Activities of Certain Preparations from Liver and Fish*

Sample	<i>Citrovorum</i> units per ml.	U. S. P. units per ml.	Chick units*	Rat units*	<i>L. lactis</i> Dorner† units per ml.
L-2	40,000	20	+++ (10)	+++ (14, 15)	116,000
L-4	34,000	15		++ (14, 15)	68,000
L-5	32,000	15	0 (10)	0 (14)	550
L-6	20,200	2		+++ (14)	43,000
L-10A	13,000	0.1		+	(15)
L-10	11,700	1		+	(15)
L-11	10,600	10		+	(15)
L-13	7,900	15	+++ (10)	+++†	29,500
L-15	4,900	10		++†	24,500
L-16	4,900	10		+++†	66,000
F-19	137		0 (10)	0†	1,000
F-21	24		+	(10)	++†

\* The figures in parentheses refer to the bibliography.

† Arbitrary units based upon a commercial preparation.

‡ Register, U. D., and Elvehjem, C. A., unpublished.

0.25  $\gamma$  per ml. failed to stimulate the growth of *L. citrovorum*. Recently Lees and Emery (16) have shown that *L. citrovorum* failed to respond to Smith's animal protein factor, while Lyman and Prescott have separated vitamin B<sub>12</sub> from the *citrovorum* factor by electrolysis (17).

The question whether the *citrovorum* factor is active clinically must be left open. The clinical potency of Samples L-5 and L-11 might suggest such activity, although it is also possible that totally unrelated materials in these relatively crude preparations may have been responsible for the observed clinical results. A low clinical potency for the *citrovorum* factor is suggested by Sample 10-A which was only 0.5 per cent as active clinically as Sample L-2, although it was 32 per cent as active as Sample L-2 for *L. citrovorum*.

## SUMMARY

1. The response of *Leuconostoc citrovorum* 8081 to a factor in liver was unaltered by the presence in the medium of oleic acid, streptogenin, tomato juice, or cobalt; these substances did not alter the response of the organism to folic acid or to thymidine.

2. The response of the organism to folic acid or to thymidine differed both qualitatively and quantitatively from its responses to preparations from liver. The most active concentrate prepared from liver was at least twice as active as thymidine.

3. Liver preparations of tested clinical potency against pernicious anemia were analyzed for their activities toward *Leuconostoc citrovorum* and the results compared with those of others for the activities of these preparations toward the chick, the rat, and *Lactobacillus lactis* Dorner. Fairly good agreement was observed between the results on the latter three species and those of clinical assays. The results with *L. citrovorum* were significantly different from those with the chick, the rat, and *L. lactis* Dorner and they were usually quite different from the results of clinical assays. Vitamin B<sub>12</sub> did not stimulate the growth of *L. citrovorum* 8081.

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# THE NUTRITION OF VARIANTS OF LACTOBACILLUS BIFIDUS\*

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The bacterial flora of the intestine of an infant that is being fed human milk is characterized by the predominance of a lactobacillus whose distinctive morphology of rods, clubs, and bifid forms has given it the name of *Lactobacillus bifidus* (1-3). The possible importance of this intestinal organism to the health of the infant makes its study one of practical significance.

Since its discovery in 1899 by Tissier (4) the nature and biological position of *L. bifidus* as a distinct entity has been the subject of controversy (5-7). The various strains used in the present study were isolated with difficulty. It is probable that the isolation procedure selected less fastidious variants or caused an adaptation of the strains normally present in the infant intestine. However, a study of the nutritional requirements of the isolated strains may serve as a starting point for the formulation of a medium nutritionally adequate for the more fastidious strains.

Since *L. bifidus* is found to predominate in the intestine of the infant receiving only human milk, it would seem that human milk affords a more beneficial medium for the propagation of this organism. Preliminary experiments revealed that the addition of milk to the culture media promoted the growth of the strains. Human milk was superior to cow's milk. A knowledge of the nutritional requirements of *L. bifidus* seemed prerequisite to the understanding of the superiority of human milk, and for this purpose that the present investigation was undertaken.

## EXPERIMENTAL

*Isolation of Strains*—Saline suspensions of stools from infants fed only human milk were streaked on liver infusion agar fortified with 10 per cent human milk. The plates were incubated anaerobically at 37°, growth of colonies being obtained usually after 48 hours. Transfers were made

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from the almost microscopic colonies to liver infusion-milk broth and carried by daily transfers. Upon primary isolation the various strains exhibited the characteristic morphology, but upon subsequent transfers the bifid characteristic gradually disappeared. This has also been the experience of other investigators (7). The loss of the bifid characteristic has been considered evidence of mutation and selection and for this reason the strains of the present study will not be termed *L. bifidus*. By varying the conditions of the isolation procedure some success was attained in maintaining several strains in the bifid form. These investigations are being continued and will be reported elsewhere.

TABLE I  
*Composition of Media*

*Liver Infusion, Double Strength*

500 gm. of beef liver and 100 ml. of H<sub>2</sub>O boiled for 2 hours, filtered, and 10 gm. of peptone, 5 gm. of NaCl, 10 gm. of lactose, and 0.1 mg. of cystine added to the filtrate. pH adjusted to 7.4 and diluted to 500 ml.

*Assay Medium, Double Strength*

K <sub>2</sub> HPO <sub>4</sub> .....	5 gm.	Thiamine HCl .....	400 γ
Glucose.....	40 "	Riboflavin.....	400 "
Sodium acetate (anhydrous)...	50 "	Ca pantothenate .....	800 "
Enzyme-hydrolyzed casein*....	10 "	Pyridoxine HCl.....	2400 "
Adenine, guanine, uracil,		<i>p</i> -Aminobenzoic acid .....	20 "
xanthine, each.....	20 mg.	Folic acid. ....	20 "
Alanine, cystine tryptophane,		Biotin .....	8 "
each.....	400 "	Salts B† .....	10 m
Asparagine.....	200 "	pH adjusted to 6.8, diluted to 1 liter	

\* N-Z-Case, Sheffield.

† 10 gm. of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 gm. of FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 gm. of NaCl, 0.337 gm. of MnSO<sub>4</sub>·2H<sub>2</sub>O in 250 ml. of H<sub>2</sub>O.

*Culture Media*—The composition of the media used in this investigation is presented in Table I. The medium used for isolation and for subculture of strains was the liver infusion medium of Boverter (8). To this basal medium, before autoclaving, the filtrate of heat-coagulated milk was added in 10 per cent concentration. The semisynthetic medium is that used by Teply and Elvehjem (9) for the assay of folic acid with *Lactobacillus casei*.

In initial experiments sodium citrate was used as the buffering agent of the medium instead of sodium acetate. Good growth of the strains was obtained when skim milk was added to the citrate medium. Upon acetone precipitation of the milk it was found that both the acetone-



soluble and the acetone-insoluble fractions were required for growth (Table II). The isolation and identification of the acetone-insoluble growth factor as calcium phosphate were explainable by the finding that the sodium citrate of the medium retarded the growth of the assay organism and that calcium salts neutralized the inhibitory effect of the citrate. Magnesium, manganese, and iron salts also reversed the citrate inhibition. MacLeod and Snell (10) have attributed the inhibitory effect of citrate to its ability to bind the essential mineral, manganese, into a complex that was not available to lactic acid bacteria. The inhibition of the *L. bifidus* strains by citrate was also found to be the result of manganese deficiency. Removal of iron and magnesium from the medium was without effect. The data of Table II demonstrate the inhibitory effect of citrate on the

TABLE II  
*Inhibition of Strain A by Sodium Citrate*

16 hours incubation.

Supplement	0.1 N acid minus blank	
	Citrate medium	Acetate medium
	ml.	ml.
1. Blank .....	0	(0.06)
2. 1 ml. skim human milk .....	7.89	13.90
3. 1 " acetone filtrate of milk...	0	13.40
4. 1 " " ppt. of milk.....	0.45	0.70
5. (3) + (4).....	8.04	13.78
6. (3) + CaCl <sub>2</sub> .....	7.50	13.88
7. (2) + 250 mg.* sodium citrate. ..		6.89

\* Amount present per tube of citrate medium.

growth of strain A. When sodium citrate of the medium was replaced by sodium acetate, supplementation with only the acetone-soluble fraction was necessary for growth.

*Assay Procedure for Growth Factors*—The activity of various substances in producing growth of the isolated strains was determined by titrimetric assay. It was established that the production of acid paralleled the growth of cells as measured by turbidity determinations and by actual count of cells. To each tube were added 5 ml. of the double strength semi-synthetic medium (acetate buffer), the test sample, and sufficient water to make a total of 10 ml. The tubes were set up in triplicate. Only two of the tubes were inoculated with the strain under investigation. The uninoculated tube served as a pH control. The inoculum consisted of 0.1 ml. of a saline suspension of the twice washed cells from an 18 hour anaerobic culture grown on liver infusion-10 per cent human milk broth. The

assay tubes were incubated in Brewer anaerobic jars at 37° for 16, 40, and 64 hours. The acid produced in each tube was determined by titration with 0.1 N NaOH, each tube being titrated to the pH of the uninoculated control.

*Growth Factor of Milk*—During the isolation of strains of *L. bifidus* it was noted that a more luxuriant growth of colonies was obtained when milk was added to the liver infusion agar. Human milk appeared superior to cow's milk. Quantitative comparison of the two milks by titrimetric assay confirmed the superiority of human milk (Table III). Skimming had little effect on the activity of either milk. When the milk was coagulated by heat at an acid pH, the activity was found with the protein precipitate. The growth factor proved to be soluble in ether. The fractionation procedure that afforded the best concentration of the growth factor is that used for the separation of unsaturated fatty acids; namely, extrac-

TABLE III

*Growth Activity of Human and Cow's Milk for Strain A*

40 hours incubation.

Milk	0.1 N acid	
	Human	Cow
	ml.	ml.
Whole, 5 ml.	22.8	12.6
" 1 "	14.2	5.7
Skim, 5 ml.	23.0	11.6
" 1 "	13.1	4.1

tion of the acidified milk with mixed ethers, extraction of ether solution with dilute alkali, precipitation with lead acetate, extraction of the lead precipitate with ethyl ether, and removal of the lead by hydrochloric acid precipitation. The details of the various steps were those of standard fat analysis (11). The finding that the growth factor of milk is associated with the unsaturated fatty acid fraction is in accordance with a number of recent reports (12, 13) that unsaturated fatty acids are growth factors for a number of lactic acid bacteria. The unsaturated fatty acids, oleic, linoleic, arachidonic, vaccenic, elaidic, and palmitoleic, and the related compounds, methyl oleate, glycerol monooleate, and Tween 80 were found to promote half maximal growth of strain A at concentrations of 250 to 750  $\gamma$  per tube. Saturated fatty acids, such as palmitic and stearic, proved inactive when tested alone and did not enhance the activity of the unsaturated fatty acids when tested in combination. It has been reported that, whereas lower concentrations of oleic acid stimulate the

growth of certain lactobacilli, higher concentrations are inhibitory (14, 15). Concentrations of oleic acid as great as 20 mg. per tube did not inhibit the *L. bifidus* mutants. The activity of oleic acid was not influenced by pH in the range of 6.0 to 7.0. Since Tween 80, a sorbitan mono-oleate polymer, is water-soluble and relatively stable, it was used for the quantitative comparison of the growth-promoting activity of human and cow's milk. 1 ml. of whole human milk was found to have an activity equivalent to 1600  $\gamma$  of Tween 80. Cow's milk was about one-fifth as active, 1 ml. being equivalent to 280  $\gamma$  of Tween 80.

Since the skimming of the milk did not decrease growth activity, it may be concluded that unsaturated fatty acid bound in the glyceride molecule is not available to the assay organism. However, the lactobacilli of the intestinal tract would have access to milk that has been digested, and for this reason a comparison of human and cow's milk was made on digested samples of the whole milk. When digested with pancreatin, both milks exhibited a great increase in growth activity as the result of the liberation of fatty acids and both milks were equally active; 0.0125 ml. of digested milk per tube gave maximal growth in a 16 hour incubation. The separation of the unsaturated fatty acid fraction from digested human milk yielded a yellow oil that was approximately 3 times more potent than Tween 80. Digested cow's milk yielded fractions of equal potency. The identity of the active constituents of the concentrates is still unknown.

*Inhibitory Factors of Digested Milk*—In comparing the activities of the digested human and cow's milk, it was observed that the addition of high concentrations of the digested cow's milk to basal medium permitted less growth than did smaller concentrations. This effect was not observed with equal concentrations of human milk (Table IV). The inhibitory substances of digested cow's milk could be concentrated by steam distillation of the acidified milk. Steam distillation of the non-inhibitory digested human milk also yielded a distillate that retarded the growth of the assay organism. It became apparent that both digested cow's and digested human milk contained inhibitory constituents but that human milk contained in addition protective factors that neutralized the effect of the inhibitor. The data of Table IV demonstrate the effect of skim human milk in reversing the inhibition by a steam distillate from digested cow's milk.

*Growth Factor of Enzymatic Digest of Casein*—The medium of Teply and Elvehjem (Table I), when fortified with a source of the unsaturated fatty acid factor, was nutritionally adequate for all of the strains. This medium contains an enzymatic digest of casein. If an acid hydrolysate of casein was substituted for the enzymatic digest, the medium no longer supported the growth of a number of the strains. This latter group, as typi-

fied by strain I, required a growth factor present in enzymatic digest of casein. The factor is not present in milk. If milk was digested with pancreatin, it became active, but the activity of the digested milk could be attributed entirely to the pancreatin added for digestion. The growth-stimulating activity of the enzymatic digest of casein may also have been introduced with the enzyme preparation. A commercial trypsin preparation also exhibited considerable activity. The factor of the enzyme preparations was not inactivated by autoclaving. Of a number of substances of microbiological interest that were tested only those compounds containing a desoxyriboside component were active; *i.e.*, desoxyribonucleic acid, thymine desoxyriboside, and guanine desoxyriboside (Table V). Ribonucleic acid was inactive and did not inhibit the activity of desoxyribonucleic acid when added at a concentration 5 times greater. Other

TABLE IV

*Effect of Skim Human Milk in Neutralization of Toxicity of Digested Cow's Milk Strain A, 16 hours incubation.*

Supplement to complete medium*	0.1 N acid
	ml.
1. Control . . . . .	12.5
2. 5 ml. digested human milk . . . . .	12.0
3. 5 " " cow's " . . . . .	2.7
4. Steam distillate of (2) . . . . .	0.5
5. " " (3) . . . . .	0.2
6. (5) + 4 ml. skim human milk . . . . .	12.3

\* 0.1 per cent Tween 80 added as source of unsaturated fatty acid.

inactive substances, tested alone and in combination, include tomato juice, glutamine, thymine, a streptogenin concentrate, pyridoxamine phosphate, ascorbic acid, and vitamin B<sub>12</sub>. These findings have been reported in a preliminary paper (15).

*Comparison of Strains*—Six strains of lactobacilli, isolated from stools of infants fed human milk, have been studied during the course of this investigation. These strains were isolated on the liver infusion-human milk agar and subcultured daily on a similar liquid medium under anaerobic conditions. The strains required anaerobiosis upon primary isolation and the cells exhibited the bifid morphology. Upon subsequent transfers the bifid morphology was replaced by rod forms and the organisms became aerobic. These findings are in agreement with those reported by the majority of investigators (7).

With the recognition of the inhibitory nature of the sodium citrate buffer of the medium used early in this study and the demonstration of a

requirement for an unsaturated fatty acid factor and a factor present in pancreatin, differences in the growth behavior of the various strains became understandable. Similarities in strains became apparent and it was possible to classify the six strains in three groups based on requirement

TABLE V  
Growth Activity of Desoxyribosides

Strain I, 16 hours incubation.

Supplement	0.02 N NaOH minus blank*
	ml.
1. Blank .....	(1.7)
2. 50 $\gamma$ desoxyribonucleic acid .....	11.3
3. 5 " " " .....	3.1
4. 5 " thymine desoxyriboside† .....	9.7
5. 1 " " " .....	5.0
6. 5 " guanine " † .....	6.5
7. 1 " " " .....	3.2
8. 0.001-1.0 $\gamma$ vitamin B <sub>12</sub> † .....	0.0
9. 100 $\gamma$ thymine .....	0.0
10. 10 mg. ascorbic acid (non-autoclaved) .....	0.0
11. 50 $\gamma$ ribonucleic acid .....	0.0
12. (3) + (11) .....	3.0
13. 50 $\gamma$ pancreatin, Viobin .....	11.5

\* 2 ml. of medium.

† We are indebted to Dr. W. Shive for the thymidine, to Dr. S. Cohen for the guanine desoxyriboside, and to Merck and Company, Inc., for the vitamin B<sub>12</sub>.

TABLE VI  
Classification of *L. bifidus* Strains

Strains	Unsaturated fatty acid requirement*	Pancreatin* requirement	Relative sensitivity to citrate
A and B .....	+	0	0
I " J .....	+	+	+
H " L .....	0	0	+

\* Acid hydrolysate of casein medium.

for unsaturated fatty acid and pancreatin factors and sensitivity to citrate inhibition (Table VI).

#### DISCUSSION

The growth of *L. bifidus* mutants on a semisynthetic medium containing enzymatically digested casein and fortified with milk was dependent on the presence of an unsaturated fatty acid factor in the milk and of a fac-

tor in enzymatic digest of casein whose activity could be replaced by desoxyribosides. Oleic acid and other unsaturated fatty acids have been shown to be growth essentials for certain strains of lactobacilli (12, 13, 16), and for other strains the biotin requirement is replaceable by unsaturated fatty acids (14, 17, 18). All of the strains isolated during the present investigation were of the former type; *i.e.*, the addition of unsaturated fatty acid was necessary for the growth of the strains in a medium containing an ample amount of biotin.

The growth activity of thymine desoxyriboside (thymidine), guanine desoxyriboside, and of desoxyribonucleic acid for strain I confirms the recent report of Kitay *et al.* (19) and of Hoff-Jørgensen (20) that the effect of thymidine in growth stimulation of lactobacilli is not a specific effect but dependent on the desoxyriboside structure. A number of investigators have reported that thymidine will replace vitamin B<sub>12</sub> as growth factor for certain lactobacilli (21). Although thymidine was growth-stimulatory for strain I, vitamin B<sub>12</sub> was totally ineffective. It has been postulated that vitamin B<sub>12</sub> functions in the synthesis of the desoxyriboside component of nucleic acid (19, 20). If this is true, the addition of vitamin B<sub>12</sub> alone will not promote synthesis of desoxyriboside by strain I. The strain of *Thermobacterium acidophilum* R<sub>26</sub> studied by Hoff-Jørgensen (20) is similar to strain I in that thymidine cannot be replaced by vitamin B<sub>12</sub>; it differs from strain I in that tryptic digest of casein cannot replace the thymidine. The microbiological assay of vitamin B<sub>12</sub> is complicated by the fact that with the organisms commonly used for assay what is measured in a given sample is the sum of vitamin B<sub>12</sub> and desoxyriboside activity. A concomitant assay with strain I, therefore, should permit a correction for the desoxyriboside activity.

The desoxyriboside activity of a partially purified trypsin preparation cautions against the common procedure of adding trypsinized casein as source of streptogenin to assay media for the stimulation of early growth of the assay organisms. In addition to streptogenin other factors may be added, and it must be emphasized that these factors are not necessarily of casein origin but may be present in the enzyme preparations.

The importance of unsaturated fatty acids for the multiplication of *L. bifidus* in the intestine of the infant may only be surmised from the results of this study. The strains employed have been considered as mutants or variants of the bacteria commonly observed in smears of infants' stools, since the anaerobic bifid organisms originally isolated gradually changed on subsequent transfers to rods that could be cultured aerobically. Preliminary investigations in this laboratory indicate that these lactobacilli can be maintained in the bifid state on a chemically defined medium.

It is planned to compare the growth requirements of bifid organisms with those of the rod-like variants.

#### SUMMARY

Anaerobic bifid lactobacilli isolated from the stools of breast-fed infants gradually changed upon subsequent subculture to rod-like aerobes. These variants of *Lactobacillus bifidus* could be grown on a semisynthetic medium containing enzymatic digest of casein if the medium were supplemented with human or cow's milk. Human milk contained a concentration of the growth factor 5 times greater than that of cow's milk. The growth factor of milk was found to be associated with the unsaturated fatty acid fraction. Unsaturated fatty acids, such as oleic, linoleic, and vaccenic, and related compounds also exhibited growth stimulation. The growth activity of whole milk was greatly increased by pancreatic digestion, and after digestion human milk and cow's milk were of equal activity. High concentrations of digested cow's milk, but not of human milk, were found to inhibit bacterial growth.

In addition to the unsaturated fatty acid factor, several of the strains required a stimulatory factor present in enzymatic digest of casein, pancreatin, or trypsin preparations. Compounds containing a desoxyriboside component were also found to be active. Crystalline vitamin B<sub>12</sub> could not replace the activity of the desoxyribosides.

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# WATER-SOLUBLE POLYSACCHARIDES OF SWEET CORN\*

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The water-soluble polysaccharides of sweet corn (*Zea mays*), particularly Golden Bantam, have been examined by different workers who arrived at somewhat different conclusions. These workers separated the water-extractable material into two fractions on the basis of solubility in 67 per cent acetic acid. The more soluble fraction, which stains red with iodine, was considered a plant glycogen, and the name "phytoglycogen" was suggested for it by Sumner and Somers (1). The more insoluble fraction, which stains blue with iodine, was considered as starch by Morris and Morris (2) and Hassid and McCready (3), while Sumner and Somers considered it a polysaccharide distinct from starch and suggested the name "glycoamylose" for it. On observing the iodine-stained glycoamylose fraction microscopically, Cameron (4) found both blue-staining and red-staining particles and, therefore, considered the fraction impure. Cameron shows that, in corns of different endosperm type, as the amount of amylose increases, the amount of glycoamylose also increases. Since the amylose-like nature of the glycoamylose has not been established and since it has been our experience that even amylose degraded to the extent that it stains red with iodine is not readily water-soluble, the present investigation was undertaken to obtain further information on the nature of glycoamylose. For comparison, the phytoglycogen fraction was also reexamined.

## EXPERIMENTAL

*Isolation of Water-Soluble Polysaccharides*—Although polysaccharides were isolated from open pollinated Golden Bantam, open pollinated Country Gentleman, and inbred line Purdue 39A sweet corn, experimental results are given only for one of these, namely Golden Bantam. All data are reported on a dry weight basis. The isolation procedure is based on that of Sumner and Somers (1) and of Hassid and McCready (3). 95 per cent ethanol was used in all precipitations.

Air-dried mature corn was finely ground and 500 gm. were extracted for 30 minutes at 15–20° with 1500 ml. of 10 per cent trichloroacetic acid. The solids were separated by centrifugation and reextracted with 750

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ml. of trichloroacetic acid solution. Both extracts were combined and clarified by passage through a supercentrifuge at 40,000 R.P.M. 2 volumes of ethanol were slowly added with rapid stirring, the precipitate was allowed to settle, and the supernatant liquid decanted. The precipitate was alternately washed with ethanol in a Waring blender and filtered until free of acid. The precipitate was dried *in vacuo* over calcium chloride. The yield was 26 per cent. On extraction with hot 85 per cent methanol (5), 0.7 per cent of fatty substances was removed. This quantity corresponds to that which can be removed under similar conditions from commercial corn-starch.

The polysaccharide mixture was further separated into two fractions. A 100 gm. portion was mixed with 1000 ml. of water, heated on a steam bath for several minutes, and then autoclaved 2 hours at 15 pounds pressure. When cold, the solution was passed through a supercentrifuge to remove precipitated protein (2.7 per cent). 2 volumes of glacial acetic acid were added, and the mixture was held overnight at 5°. The supernatant solution containing phytoglycogen (Fraction 2) was decanted. The precipitated glycoamylose (Fraction 1) was washed with ethanol in a Waring blender, filtered, dissolved in 500 ml. of water, adjusted to pH 6.5 with sodium hydroxide, and precipitated by the addition of 1.5 volumes of ethanol. The precipitate was washed with several portions of ethanol in a Waring blender and dried *in vacuo* over calcium chloride. The yield was 82 per cent of the soluble polysaccharide mixture.

Fraction 2 was isolated from the supernatant solution by precipitation with 1.5 volumes of ethanol. It was then treated in the same manner as Fraction 1 except that 2 volumes of ethanol were used in the final precipitation. The yield was 15 per cent of the soluble polysaccharide mixture.

Based on the original sweet corn, Fraction 1 was obtained in 21 per cent yield and Fraction 2 in 4 per cent yield.

In the separation of the two fractions, storage overnight at 5° has no adverse effect, since identical results are obtained if the precipitate is worked up immediately.

*Purification of Fraction 1*—In the solid state or in solutions of about 1 per cent or more concentration, Fraction 1 is colored blue by iodine. The blue-staining portion is removed by adsorption on cotton (6). Thus, if a 1 per cent solution of the carbohydrate is passed through a column of previously washed U. S. P. absorbent cotton, the effluent yields a lavender color with iodine, while the blue-staining material is bound near the top of the column. Its position can be located by extruding the column and streaking with iodine solution. About 3 per cent of the fraction is adsorbed by the cotton. This was indicated by passing 400 ml. of a 1 per

cent solution of Fraction 1 through a cotton column  $34 \times 200$  mm. After the column was washed with 300 ml. of water, the washings and filtrate were combined, concentrated, and the solids determined by evaporation of 10 ml. portions on Filter-Cel (7). The recovery was 97 per cent.

Larger amounts of purified Fraction 1 were prepared by passing 300 ml. of 10 per cent solution through a column of cotton  $40 \times 600$  mm., washing with 600 ml. of water, concentrating the combined solutions *in vacuo*, and precipitating with ethanol.

*Preparation of Limit Dextrins*—Limit dextrins were prepared essentially according to the procedure of Hodge, Montgomery, and Hilbert (8) except for the added precaution of inactivating any  $\alpha$ -amylase present in

TABLE I  
*End-Group Assay of Limit Dextrins by Periodate Oxidation*

Time, hrs. ....	Dextrin from total water-soluble Golden Bantam sweet corn polysaccharide						Dextrin from white potato amylopectin, 0.4 gm. sample*		
	0.2 gm. sample*			0.4 gm. sample*					
	73	120	168	73	120	168	73	120	168
	moles	moles	moles	moles	moles	moles	moles	moles	moles
Glucose residue per mole formic acid....	9.0	8.0	7.4	8.4	7.8	7.5	13.0	12.0	12.0
Periodate per mole glu- cose residue, found.	1.15	1.19	1.20	1.05	1.10	1.13	1.02	1.07	1.10
Periodate per mole glucose residue, cal- culated....	1.11	1.13	1.14	1.12	1.13	1.13	1.08	1.08	1.08

\* The sample weight was dissolved in 100 ml. of 5 per cent potassium chloride solution; 10 ml. of water and 10 ml. of 0.3 M sodium periodate solution were added.

the wheat  $\beta$ -amylase extract by lowering the pH to 3.0 for 2 hours at  $30^\circ$  (9). The enzyme brought about a 52 per cent conversion of white potato amylopectin; Hodge *et al.* (8) report 54 per cent. For Fractions 1 and 2, the limits of hydrolysis were 47 and 45 per cent, respectively, and were reached in 2 days, whereupon they remained constant for 4 days, even when fresh enzyme solution was added each day. In the isolation of the limit dextrins, ethanol was employed for precipitation and washing, since the limit dextrins were found to be soluble in 50 per cent ethanol. Even so, the limit dextrin of Fraction 2 was partially soluble, for only 56 per cent was recovered. On the other hand, 92 per cent of the limit dextrin of Fraction 1 was recovered.

*Periodate End-Group Assay*—The method of Hirst *et al.* (10) was used without change, as it seems to work well with amylopectin-like materials.

Results obtained on the limit dextrin of the total water-soluble fraction of sweet corn and the limit dextrin of white potato amylopectin (Table I) show that the formic acid production levels off satisfactorily and that the end-group value obtained is independent of sample size. The amount of periodate consumed was also determined (11). The values found are in good agreement with the theoretical amounts for materials with the end-group assays shown. The value of 12 found for the amylopectin limit dextrin of white potato is consistent with the end-group assay of 24 to 26 for white potato amylopectin (12) and the extent of hydrolysis (52 per cent) for the amylopectin.

Results obtained by this method of end-group assay for Fraction 1 and 2 and their limit dextrans are shown in Table II.

TABLE II  
*Properties of Fractions 1 and 2 from Golden Bantam Sweet Corn*

Polysaccharide	Color with iodine	Cupric chloride pattern	Appearance of 1 per cent water solution	Conversion to maltose by wheat $\beta$ -amylase	Periodate end-group assay
				<i>per cent</i>	
Crude Fraction 1	Blue	Glycogen type	Opalescent		
Purified Fraction 1	Lavender		"	47	12
Fraction 1 limit dextrin	Purple		"		7
Fraction 2	Purple-gray	Glycogen type	Clear	45	11
" 2 limit dextrin	None		"		7

*Determination of Color with Iodine*—As the color of these polysaccharide solutions varies a great deal with the concentration of the carbohydrate and of the iodine, all colors were determined by adding 2 drops of 0.01 N iodine solution to 5 ml. of a 1 per cent solution of the carbohydrate.

#### DISCUSSION

Different workers have not always obtained the same yields of Fractions 1 and 2 from Golden Bantam sweet corn. This variation is shown in Table III. The differences are probably due to environmental modifications of the quantity and nature of the water-soluble extract. Cameron (4) has shown that such differences may be expected among corns of various endosperm types.

Examination of the crude Fraction 1 shows that it stains blue with iodine only in the solid state or in solutions of concentration greater than

1 per cent. It does not take up an appreciable amount of iodine during potentiometric titration according to the established procedure (13) and gives no spectral absorption typical of the amylose-iodine complex (14). Likewise, aqueous solutions of the fraction fail to precipitate when subjected to the butanol fractionation procedure (15). On the other hand, a small amount of blue precipitate forms when the aqueous solution is treated with iodine. On passage of an aqueous solution of the fraction through a column of cotton (6), the blue-staining material, which amounts to 3 per cent of the fraction, is preferentially adsorbed by the cotton and can be detected as a zone by streaking the extruded chromatogram with iodine. These facts indicate the presence of a small amount of amylose or amylose-like material as a constituent of the fraction. After purification by passage through cotton, Fraction 1 stains lavender with iodine, produces opalescent solutions, and has an end-group assay of 12. It is

TABLE III  
*Yields of Fractions 1 and 2 from Golden Bantam Sweet Corn*

Investigators	Fraction 1	Fraction 2
	<i>per cent</i>	<i>per cent</i>
Morris and Morris (2) . . . . .		10-13
Hassid and McCready (3) . . . . .	10-15	3- 5
Sumner and Somers (1) . . . . .	10	20
Meyer and Fuld (16) . . . . .	-	5-10*
Dvonch and Whistler . . . . .	21	4

\* Based on fresh corn.

hydrolyzed to the extent of 47 per cent by wheat  $\beta$ -amylase to give a limit dextrin staining purple with iodine and having an end-group assay of 7. From these observations, it is evident that the name "glycoamylose" is unsuited to Fraction 1 and particularly to purified Fraction 1, because the fraction is quite unlike amylose.

Fraction 2 differs from that of Morris and Morris by staining a faint purple-gray with iodine and by producing clear water solutions. It is hydrolyzed by wheat  $\beta$ -amylase to the extent of 45 per cent, confirming the recent work of Meyer and Fuld (16), which is in disagreement with that of Morris (17). It has an end-group assay of 11, and its limit dextrin one of 7.

Identical cupric chloride crystallization patterns (18) are given by crude Fraction 1 and Fraction 2 and by glycogen. The limit dextrin of the total water-soluble polysaccharides also gives the same pattern. Corn amylopectin and its limit dextrin give the dextrin pattern reported by Morris and Morris (2).

From these data, summarized in Table II, it would seem that the purified Fraction 1 is structurally similar to polysaccharide Fraction 2, especially since Hassid and McCready (3) report similar molecular weights for their methyl ethers.

One of the fundamental structural differences between glycogen and amylopectin is the greater frequency of branching in the former substance. From the data given here, it is apparent that the degree of branching for the water-soluble sweet corn fractions corresponds to that found in glycogen. The fractions differ from glycogen in their color with iodine.

While practically all of the water-soluble polysaccharides of various sweet corns, such as Golden Bantam, may be considered glycogen-like in their degree of branching, there seems to be no advantage gained in designating them as glycogen. The term glycogen has long been used in the biochemical literature to designate the branched glucan which is the polysaccharide reserve of animals and yeasts, and use of the term may well be restricted to this designation. Various types of glycogen occur which may differ slightly in their structure. As Meyer (19) has pointed out, glycogen is not a pure chemical entity, but is rather a biological conception. Likewise, amylopectins of different structural types occur in plants. It is quite likely that further work will bring to light amylopectins with quite different degrees of branching. Consequently, it might be more logical to consider the water-soluble glucans of sweet corn as simply highly branched amylopectins.

The authors wish to thank Mr. J. E. Hodge and Dr. R. J. Dimler of the Northern Regional Research Laboratory for the samples of corn amylopectin and corn amylopectin limit dextrin used in this work.

#### SUMMARY

The two water-soluble polysaccharides of sweet corn were studied and were found to be similar in their properties. Both of them are amylopectin- or glycogen-like. It is suggested that they be considered amylopectins.

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# CREATINURIA DURING RECOVERY FROM AMINOPTERIN-INDUCED FOLIC ACID DEFICIENCY IN THE MONKEY\*

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Several reports (1-3) have indicated that folic acid<sup>1</sup> is involved in glycine metabolism. Since glycine is a precursor of both creatine and purines, it seemed desirable to study the effects of folic acid deficiency on the excretion of creatine, creatinine, uric acid, and allantoin by rhesus monkeys. Day and coworkers (4) have shown that monkeys require folic acid in the diet; however, it may require many months to produce the deficiency state by dietary means alone. For these studies we resorted to the use of the folic acid antagonist 4-aminopteroylglutamic acid (aminopterin). Studies were made of urinary nitrogen partition in monkeys during the induced deficiency and while recovering from aminopterin treatment, of monkeys given aminopterin until death, and of monkeys receiving large doses of folic acid without previous aminopterin treatment.

Several control animals were studied to measure the normal urinary partition, since the literature contains very few such data for the rhesus monkey. The average values found, as mg. per kilo of body weight per day, were uric acid 1.7, allantoin 8.4, and creatinine 34.7. These normal monkeys were found to excrete only insignificant amounts of creatine, and usually none at all. During recovery from aminopterin treatment, however, a very high creatinuria was observed. The excretion of uric acid and allantoin was greatly elevated. The excretion of these nitrogenous constituents returned to normal levels after a period of time.

## EXPERIMENTAL

Sexually immature, male, rhesus monkeys, weighing from 2.5 to 4.0 kilos each, were housed in steel metabolism cages equipped with stainless steel funnel-shaped trays for the collection of urine. They were fed the

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<sup>1</sup> Pteroylglutamic acid.

diet described by Day and Totter (5). Aminopterin was injected intramuscularly in doses of from 0.4 to 0.6 mg. per day. After a period of from 5 to 10 days the monkeys exhibited symptoms of severe folic acid deficiency, as evidenced by diarrhea and leucopenia. The aminopterin was then discontinued and the animals were injected with 15 mg. of folic acid daily until recovery was complete. Preliminary experiments indicated that discontinuation of aminopterin was necessary for recovery,

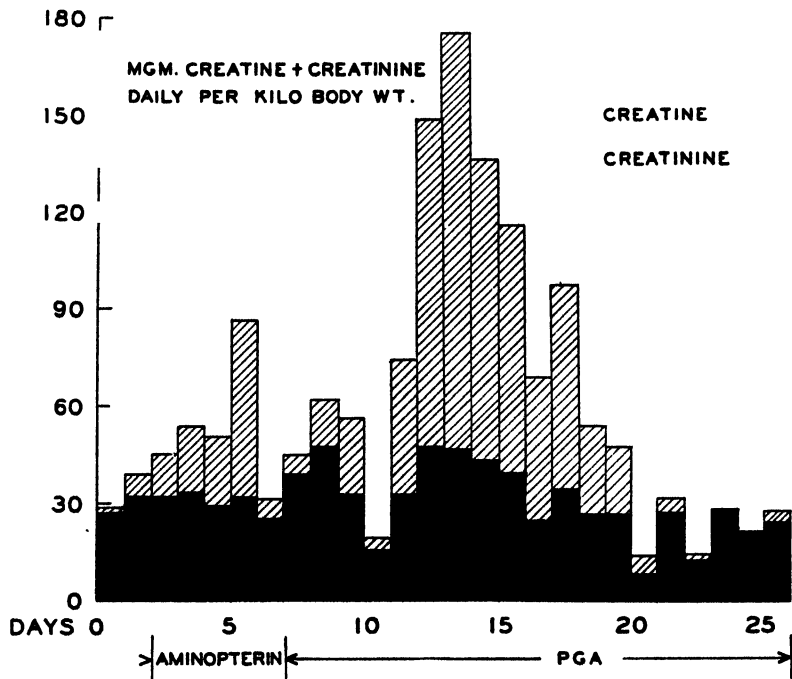


FIG. 1. Daily excretion of creatine and creatinine per kilo of body weight during aminopterin treatment and during recovery therefrom with folic acid (PGA). Monkey 172.

since the animals would not respond adequately to folic acid in the dosages used while receiving aminopterin. Other monkeys were not permitted to recover but were given aminopterin until death; controls were injected with 15 mg. of folic acid daily without having been previously treated with aminopterin. The 24 hour urine samples were collected under toluene and the various nitrogenous constituents determined on appropriate aliquots. Creatine and creatinine were determined by the method of Folin (6), uric acid by the method of Brown (7), and allantoin by the Young-Conway procedure (8).

## RESULTS AND DISCUSSION

The results of a typical experiment (Monkey 172) are presented in Fig. 1. This animal was given a total of 3.0 mg. of aminopterin over a 5 day period. The white blood cell count fell to 4600 cells per  $\mu$ l. on the 7th day of the experiment, at which time aminopterin was discontinued. After folic acid therapy the white cell count rose rapidly and reached a value of 32,000 cells per  $\mu$ l. on the 14th day of the experiment. The monkey refused to eat the diet during the first 5 days of the experiment, and it was supposed that the mild creatinuria observed at that time may

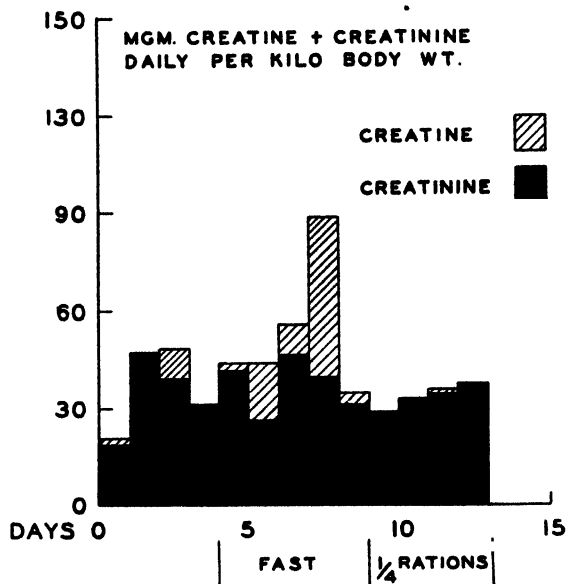


FIG. 2. Daily excretion of creatine and creatinine per kilo of body weight by a normal monkey (No. 167), showing the effects of complete and partial inanition.

have been due to inanition. Accordingly a normal monkey (No. 167, Fig. 2) was fasted 5 days and then given one-fourth rations for 6 days more. Creatinuria developed and on the 4th day of the fast the creatine excretion was approximately equal to the creatinine excretion. However, when the animal was placed on one-fourth rations, the creatinuria completely disappeared, indicating that total inanition was necessary to evoke the creatinuria. It would seem then that the creatinuria observed in Monkey 172 (Fig. 1) on the 5th day of the experiment may have been due to inanition. Minor changes in the diet were made on the 5th day, rendering it more palatable for the monkey, and it may be noticed that the creatinuria almost disappeared. During recovery of this monkey it may be seen that

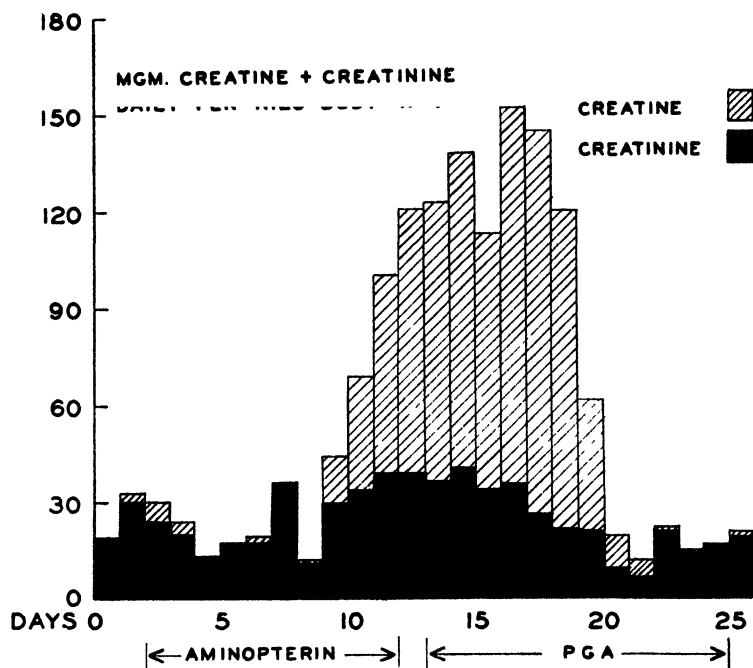


FIG. 3. Daily excretion of creatine and creatinine per kilo of body weight during aminopter-in-induced folic acid deficiency and during recovery with folic acid (PGA). Monkey 173.

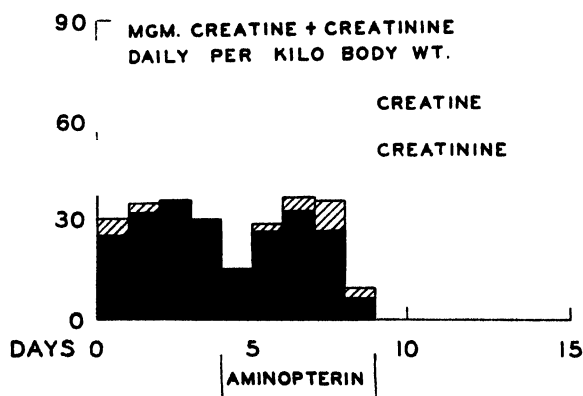


FIG. 4. Daily excretion of creatine and creatinine per kilo of body weight during a fatal experiment. Monkey 169.

a marked creatinuria developed. Creatine excretion reached a peak on the 7th day of folic acid treatment, at which time the total creatinine coefficient was 176. This creatinuria was not at the expense of creatinine.

The creatinuria rapidly diminished and had almost disappeared by the 13th day of folic acid treatment. A similar experiment is shown in Fig. 3.

Fig. 4 gives typical results obtained by injecting a monkey with aminopterin until death. No significant creatinuria was observed, indicating that the creatinuria discussed above was the result of recovery from aminopterin treatment by the use of folic acid.

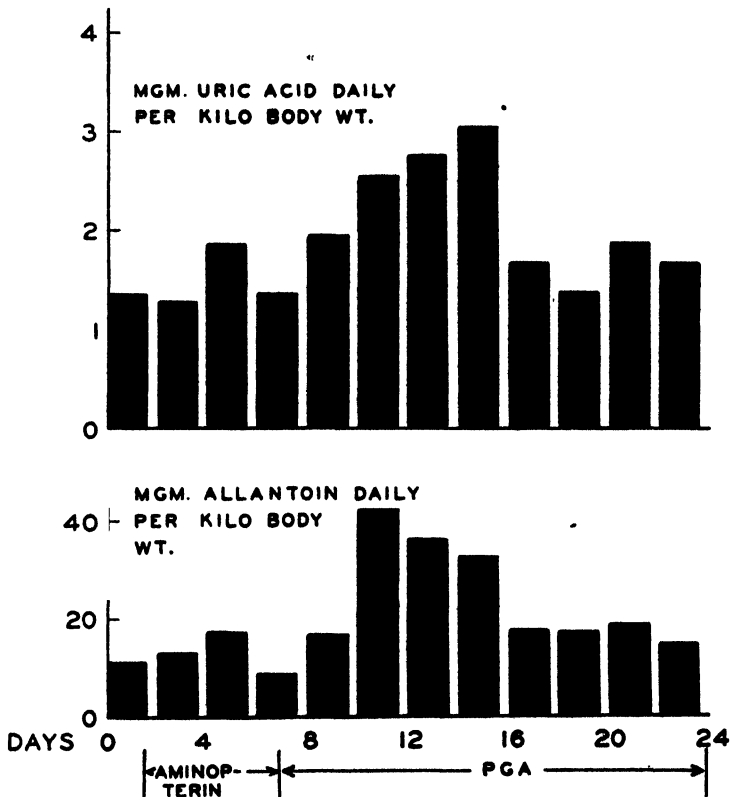


FIG. 5. Daily uric acid and allantoin excretion per kilo of body weight during and following aminopterin-induced folic acid (PGA) deficiency. Monkey 172. Averages of 2 day values.

Typical results obtained by determination of uric acid and allantoin are presented in Fig. 5. The excretion of allantoin was greatly elevated during recovery from aminopterin treatment and reached a peak the 5th day of folic acid therapy. At this time the excretion of allantoin was about 4 times as great as the control values. Allantoin excretion had returned to normal levels by the 9th day of folic acid treatment. The excretion of uric acid followed a similar pattern, reaching a peak at about

the 8th day of folic acid treatment and then rapidly returning to normal. Data from a monkey given aminopterin until death are presented in Fig. 6; it may be noted that excretion of uric acid and allantoin was not affected.

The injection of 15 mg. of folic acid daily into normal monkeys had no consistent effect on the excretion of uric acid and allantoin and did not evoke creatinuria.

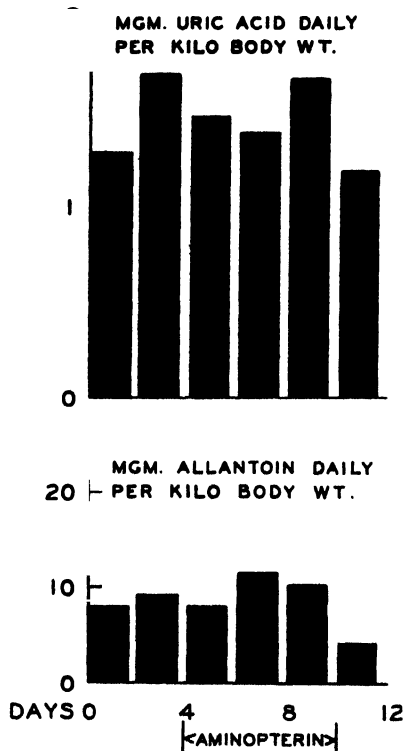


FIG. 6. Daily excretion of uric acid and allantoin per kilo of body weight in a fatal experiment. Monkey 170.

The elevated uric acid and allantoin excretions observed during recovery of these monkeys from aminopterin treatment may reflect an accelerated purine synthesis. These animals were rapidly producing white blood cells, and an elevated uric acid excretion is known to be associated with leucemia in man.

Details of peripheral blood and bone marrow cytology will be reported in detail elsewhere. It is of interest to point out here, however, that the point of maximum creatine, uric acid, and allantoin excretion coincided approximately with the point of maximum white cell response.

The creatinuria observed in these experiments occurred at a time when the monkeys were recovering and were maintaining body weight. It would seem then that it could not be attributed merely to loss of body creatine. It is possible that this creatinuria was the result of a greatly accelerated synthesis of creatine, and that this accelerated creatine synthesis is associated with recovery from aminopterin treatment. It might then follow that during the aminopterin-induced deficiency state creatine synthesis was curtailed.

#### SUMMARY

Juvenile male rhesus monkeys were housed in individual steel metabolism cages equipped with funnel-shaped stainless steel pans for urine collections. Average normal values for the following nitrogenous urine constituents, expressed as mg. of substance per kilo of body weight in 24 hours, were found to be uric acid 1.7, allantoin 8.4, and creatinine 34.7. Creatine excretion was negligible.

Certain animals were given a folic acid-deficient diet. Aminopterin was given parenterally in sufficient amount (0.4 to 0.6 mg. per day for 5 to 8 days) to produce diarrhea and profound leucopenia. A mild creatinuria developed in some of the monkeys during this period. The monkeys were then given large doses of folic acid. The recovery phase was characterized by a marked increase in urinary output of uric acid and allantoin, and by a very high creatinuria; one monkey showed a maximum excretion of 140 mg. of creatine per kilo of body weight in 24 hours. The creatinuria greatly exceeded that produced in a normal control monkey by complete withdrawal of food. The peak of creatine excretion coincided approximately with the point of maximum white cell response. The administration of large doses of folic acid to a normal monkey did not alter the amounts of the urinary nitrogen constituents measured.

The data suggest that folic acid is involved in creatine formation.

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# PRODUCTS OF THE OXIDATION OF GLYCOLIC ACID AND *l*-LACTIC ACID BY ENZYMES FROM TOBACCO LEAVES\*

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An enzyme catalyzing the oxidation of *l*- $\alpha$ -hydroxymonocarboxylic acids has been reported earlier (1). It oxidizes glycolic acid (optimum pH range 7.8 to 8.6) about three times as rapidly as *l*-lactic acid (optimum pH 7.6). The enzyme is of particular interest because of its high activity, its wide distribution among green plants, its absence from etiolated plants, its unusually low energy of activation, and because it may function as a terminal oxidase in plants. Glycolic acid, though apparently present in many plants, has not been associated with any physiological function of the plants.

In the present investigation, a procedure has been worked out for further purification of the enzyme ( $Q_{O_2}$  (N) about 50,000). This enzyme preparation oxidized glycolic acid, via glyoxylic acid, to formic acid and  $CO_2$ . Lactic acid is oxidized to pyruvic acid.

## EXPERIMENTAL

**Methods**—Measurements were made in Warburg respirometers at 30°. Values for R.Q. were determined in Warburg flasks with a simple and a Siamese side arm ((2), p. 112). One sac of the Siamese side arm contained a filter paper wick and 0.5 ml. of 2 N sodium hydroxide (previously treated with excess barium chloride to remove sodium carbonate) or 0.5 ml. of saturated barium hydroxide; the other sac contained 0.5 ml. of 4 N hydrochloric acid. The course of the oxygen uptake could be followed, and the total  $CO_2$  liberated could be measured at any time by tipping the acid into the alkali and then mixing the entire contents of the flask. The pH thus was lowered to 1.0 or less, the enzyme was inactivated, and bound  $CO_2$  was released. In a control flask, the acid was added at the beginning of the run to measure the initial bound  $CO_2$  in the solutions. In some experiments the combined contents of the flask were brought only to pH

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5.0; almost all of the bound  $\text{CO}_2$  is released under these conditions but decomposition of acid-labile compounds is minimized.

In experiments designed for the isolation of the products of the reactions, Warburg flasks of 110 ml. volume were used. The flasks were filled with tank oxygen, and mercury was used as the manometer fluid.

*Materials*—Commercial glycolic acid and *dl*-lactic acid were used for large scale runs.

Glyoxylic acid was prepared by the oxidation of *n*-dibutyl tartrate with  $\text{Pb}_2\text{O}_4$  in the presence of glacial acetic acid or with lead tetraacetate followed by careful saponification of the butyl glyoxylate.<sup>1</sup> The acid was isolated as the dihydrated barium salt. Although the formula for glyoxylic acid is written as  $\text{CHO}\cdot\text{COOH}$ , it actually exists as the monohydrate,  $\text{CH}(\text{OH})_2\cdot\text{COOH}$ , and the water of hydration cannot be removed by heating without further decomposing the acid (3). Thus glyoxylic acid may be considered as an  $\alpha$ -hydroxy acid, and its oxidation by the enzyme in question is not surprising.

The semicarbazone of glyoxylic acid could be prepared from the acid and semicarbazide, but it was more convenient to form this derivative by refluxing chloral hydrate and semicarbazide hydrochloride for 30 minutes (4). The semicarbazone was readily recrystallized from hot water.

Tobacco leaves were used exclusively as the source of the enzyme. The enzyme was concentrated by means of precipitation with sodium sulfate and ammonium sulfate, as already reported (1); such preparations are designated as salt-precipitated enzyme. These have a  $Q_{O_2}$  (N) of about 2000, contain about 88 per cent of the original activity of the tobacco sap, and can be dialyzed against water at 5° overnight without loss of activity. During dialysis, some protein material precipitates and can be removed by centrifugation. The dialyzed solution has a pH of about 6.0; after it is adjusted to pH 7.5 to 8.0 it may be stored frozen for months without appreciable loss of activity. If the dialyzed solution is lyophilized and stored under vacuum, it also retains its activity. Although the lyophilized powder contains only about 62 per cent of the total activity of the salt-precipitated enzyme, the enzyme is greatly purified by this treatment. When the lyophilized material is triturated with cold buffer at pH 8.0, the greater part of the material does not dissolve and can be removed by centrifugation from the soluble enzyme. The supernatant has a  $Q_{O_2}$  (N) of about 50,000; it is designated as the lyophilized enzyme.

### Results

*Oxidation of l-Lactic Acid*—When *l*-lactic acid is used as the substrate for the salt-precipitated enzyme, 1 atom of oxygen is taken up per mole

<sup>1</sup> Lauer, W. M., personal communication.

of lactic acid oxidized. R.Q. values are low, varying from 0.2 to 0.3. The uptake of 1 atom of oxygen suggests the formation of pyruvic acid. To test this, 5 ml. of *dl*-lactic acid in about 500 ml. of reaction mixture were oxidized at pH 8.0 in phosphate buffer by the salt-precipitated enzyme. After 8 hours, concentrated hydrochloric acid was added to bring the pH to 2.0. The precipitated protein was centrifuged off and a 2,4-dinitrophenylhydrazone derivative was prepared, redissolved in ethyl acetate, extracted with 10 per cent sodium carbonate, and reprecipitated by acidification. The melting point was 221° (corrected); the melting point of the 2,4-dinitrophenylhydrazone of authentic pyruvic acid and the mixed melting point also were 221° (corrected). The acid equivalent was 269; theory, 268. Evidently the main product of the oxidation of *l*-lactic acid by the enzyme from tobacco leaves is pyruvic acid. Pyruvic acid did not support oxygen uptake nor carbon dioxide output by the salt-precipitated enzyme. The small but significant amount of carbon dioxide released when lactic acid was oxidized has not been explained.

*Oxidation of Glycolic Acid*—In the enzymatic oxidation of glycolic acid, 1 mole of oxygen is consumed per mole of glycolic acid oxidized. This value is constant regardless of the degree of purification of the enzyme. In contrast to the constancy of oxygen uptake, CO<sub>2</sub> output per unit of substrate was variable. Each preparation of tobacco sap or salt-precipitated enzyme gave a different R.Q. The R.Q. values generally ranged from 0.2 to 0.4 for the tobacco sap and from less than 0.1 to around 0.6 for the salt-precipitated or salt-precipitated and dialyzed enzyme. The R.Q. of the highly purified lyophilized enzyme, however, was consistently  $1.00 \pm 0.02$ .

*Isolation of Derivative of Glyoxylic Acid*—The 2,4-dinitrophenylhydrazone of glyoxylic acid was isolated from the enzymatic oxidation of 2 to 3 gm. of glycolic acid in a volume of about 200 ml. The isolation of a glyoxylic acid derivative was possible only when the enzymatic oxidation of glycolic acid did not run to completion, for when the total oxygen uptake corresponded to 1 mole of oxygen per mole of glycolic acid, no hydrazone could be isolated. This indicated that glyoxylic acid was an intermediate and not an end-product. The method of isolation and purification was the same as for the 2,4-dinitrophenylhydrazone of pyruvic acid, except that the glyoxylic acid derivative was recrystallized from 50 per cent ethyl alcohol. Its melting point was 188–190°, as was a mixed melting point with authentic glyoxylic acid 2,4-dinitrophenylhydrazone. Microanalysis showed H 2.61 per cent, and C 38.19 per cent; theory, 2.41 per cent and 38.40 per cent respectively.

Compounds such as hydroxylamine, semicarbazide, phenylhydrazine, 2,4-dinitrophenylhydrazine, and ethylenediamine block the oxidation of

glyoxylic acid. Manometric measurements showed that a final concentration of 0.1 M semicarbazide blocked the oxidation of 0.0067 M glycolic acid after 1 atom of oxygen had been taken up per mole of glycolic acid; no  $\text{CO}_2$  was released. This is only half the total oxygen uptake observed in the normal glycolic acid oxidation.

In large scale runs, glycolic acid was oxidized by salt-precipitated enzyme in the presence of semicarbazide. At the end most of the proteins were removed by adjusting to pH 1, and, upon standing overnight, a semicarbazone was precipitated. It was recrystallized from hot water after clarification with activated charcoal. The melting point was 214–216° (decomposition), as was the mixed melting point with authentic glyoxylic acid semicarbazone. The neutralization equivalent was 130; theory for glyoxylic acid semicarbazone, 131. A wet carbon analysis indicated 27.1 per cent carbon; theory, 27.5 per cent. The semicarbazone of the enzymatically synthesized glyoxylic acid and the semicarbazone of glyoxylic acid formed by the treatment of chloral hydrate with semicarbazide each yielded a benzyl ester which melted without decomposition at 223–225°.

*Oxidation of Glyoxylic Acid to Formic Acid*—In the over-all oxidation of a mole of glycolic acid by the lyophilized enzyme, 1 mole of oxygen is taken up and 1 mole of  $\text{CO}_2$  is released. As demonstrated, glyoxylic acid is first formed and this reaction requires 0.5 mole of oxygen per mole of glycolic acid. When glyoxylic acid is added as a substrate to tobacco sap or to the various enzyme preparations, it is oxidized. This always is accompanied by the uptake of 0.5 mole of oxygen, but only with the lyophilized enzyme does the amount of  $\text{CO}_2$  released approach 1 mole per mole of glyoxylic acid oxidized. This discrepancy in the evolution of  $\text{CO}_2$  among the various enzyme preparations is the same as that observed when glycolic acid is the substrate.

In an attempt to determine the end-products of the oxidation of glycolic acid by the lyophilized enzyme and to find which carbon atom of the molecule yielded each product, experiments were run with labeled glycolic acid, either  $\text{C}^{14}\text{H}_2\text{OH-COOH}$  or  $\text{CH}_2\text{OH-C}^{14}\text{OOH}$ . 100 mg. of  $\text{C}^{14}\text{H}_2\text{OH-COOH}$  were dissolved in 10 ml. of phosphate-borate buffer (phosphate and borate were each 0.1 M), and the pH was adjusted to 8.2. The buffer solution containing substrate was transferred quantitatively to a large Warburg flask with 9 ml. of water. 10 ml. of the lyophilized enzyme solution were placed in a side arm, 1 ml. of 20 per cent potassium hydroxide was placed in a removable side arm, and the system was flushed with oxygen. After 7 hours, the oxygen uptake corresponded to the oxidation of 99 per cent of the glycolic acid. 1 ml. of 4 N hydrochloric acid then was added from a side arm to liberate  $\text{CO}_2$  from the solution, and the flask was shaken 30 minutes longer. The potassium hydroxide solution

from the flask was treated with saturated barium hydroxide, and the barium carbonate removed by centrifugation was washed once with  $\text{CO}_2$ -free water and twice with methanol. Its radioactivity was measured; per mg. of carbon it gave 0.15 count per second.

The contents of the Warburg flask were heated to  $80^\circ$ , coagulated protein was removed, and the solution adjusted to pH 6 and analyzed for formic acid by oxidation with mercuric chloride (5). The oxidation was run in a wet carbon apparatus similar to that described by McCready and Hassid (6). A 12 ml. sample containing less than 10 mg. of formic acid, 1 ml. of saturated sodium acetate, and 0.2 ml. of 3 N hydrochloric acid was placed in the reaction vessel and  $\text{CO}_2$  was swept out with  $\text{CO}_2$ -free air. 2.0 ml. of mercuric chloride reagent (10 gm. of mercuric chloride plus 15 gm. of sodium chloride made to 100 ml. and filtered) were added. The reaction vessel then was heated in a boiling water bath for 2 hours while  $\text{CO}_2$ -free air was passed slowly through; the  $\text{CO}_2$  evolved was captured in a Vigreux column containing standard barium hydroxide, the excess of which was titrated. Analysis of known samples of 2 to 10 mg. of formic acid gave 98 to 102 per cent recovery. When two 100 mg. samples of glycolic acid were oxidized by the lyophilized enzyme, they yielded 59.4 and 60.5 mg. of formic acid. These yields represent 98.5 and 100.0 per cent recovery of 1 mole of formic acid from each mole of glycolic acid oxidized.

As soon as the excess barium hydroxide was titrated to the end-point of phenolphthalein, excess barium hydroxide was added, the barium carbonate was removed, and its radioactivity determined as described above. Per mg. of carbon the barium carbonate registered 30.4 counts per second. The solution remaining in the wet carbon apparatus after the oxidation of formic acid contained no detectable radioactivity. Therefore 99.5 per cent of the  $\text{C}^{14}$  from  $\text{C}^{14}\text{H}_2\text{OH}\cdot\text{COOH}$  was present in the formic acid. In a similar experiment, when  $\text{CH}_2\text{OH}\cdot\text{C}^{14}\text{OOH}$  was used as a substrate, all of the  $\text{C}^{14}$  was accounted for in the  $\text{CO}_2$  evolved and none remained in the formic acid.

The identity of formic acid as an end-product of glycolic acid oxidation by the lyophilized enzyme was substantiated by determination of the Duclaux constants and the formation of a solid derivative of the oxidation product. The three Duclaux values for the acid produced were 3.3, 4.1, and 4.5. The *p*-bromophenacyl ester of the acid melted at  $140\text{--}141^\circ$ , and the mixed melting point with *p*-bromophenacyl formate was the same (7).

*Inhibition of Oxidation of Glyoxylic Acid*—Oxalic acid at 0.1 M final concentration caused 88 per cent inhibition of the rate of oxidation of 0.0067 M glyoxylic acid by the salt-precipitated enzyme. Under the same

conditions the initial rate of oxidation of glycolic acid at 0.0033 M concentration was inhibited 27 per cent (Fig. 1). The oxidation of glycolic acid in the presence of 0.1 M oxalic acid proceeds rapidly until one-half of the theoretical amount of oxygen has been taken up; after this there is severe inhibition of any further oxidation. No  $\text{CO}_2$  is released. This be-

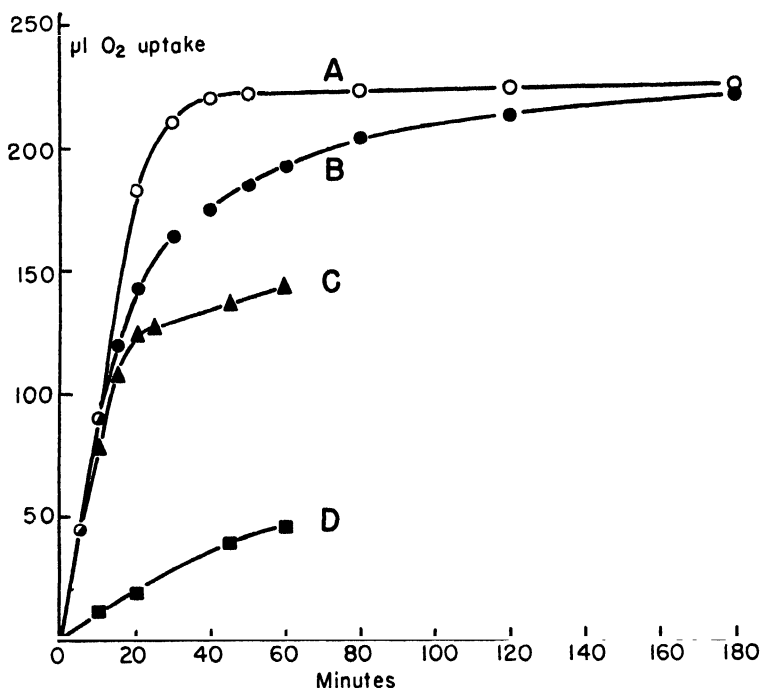


FIG. 1. Inhibition of the oxidation of glyoxylic acid by oxalic acid. The flasks contained Curve A, 0.5 ml. of 0.02 M glycolic acid; Curve B, 0.5 ml. of 0.04 M glyoxylic acid; Curve C, 0.5 ml. of 0.02 M glycolic acid plus 1 ml. of 0.3 M oxalic acid; and Curve D, 0.5 ml. of 0.04 M glyoxylic acid plus 1 ml. of 0.3 M oxalic acid. In addition each flask contained 1 ml. of phosphate-borate buffer, 0.2 ml. of salt-precipitated enzyme, and water to bring the total volume to 3.0 ml. The pH was adjusted to 8.2.

havior corresponds to the oxidation of glycolic acid to glyoxylic acid and the inhibition of further oxidation of glyoxylic acid.

Mesoxalic acid, also at a concentration of 0.1 M, caused a 42 per cent inhibition of the rate of oxidation of glyoxylic acid. Hydrazine, semicarbazide, and ethylenediamine completely inhibited glyoxylic acid oxidation; they react rapidly with keto compounds. Ascorbic acid at 0.0033 M concentration caused a 44 per cent inhibition of the rate of oxidation at pH 8.0 of 0.0067 M glycolic acid. At a lower pH where glycolic acid oxidation was not optimum, ascorbic acid completely inhibited oxidation of glycolic acid.

**Optimum pH**—As has been observed previously, the rate of oxidation of glycolic acid is about constant between pH 7.8 and 8.6. The optimum pH for glyoxylic acid oxidation is 7.7 (Curve A, Fig. 2). As oxalic acid

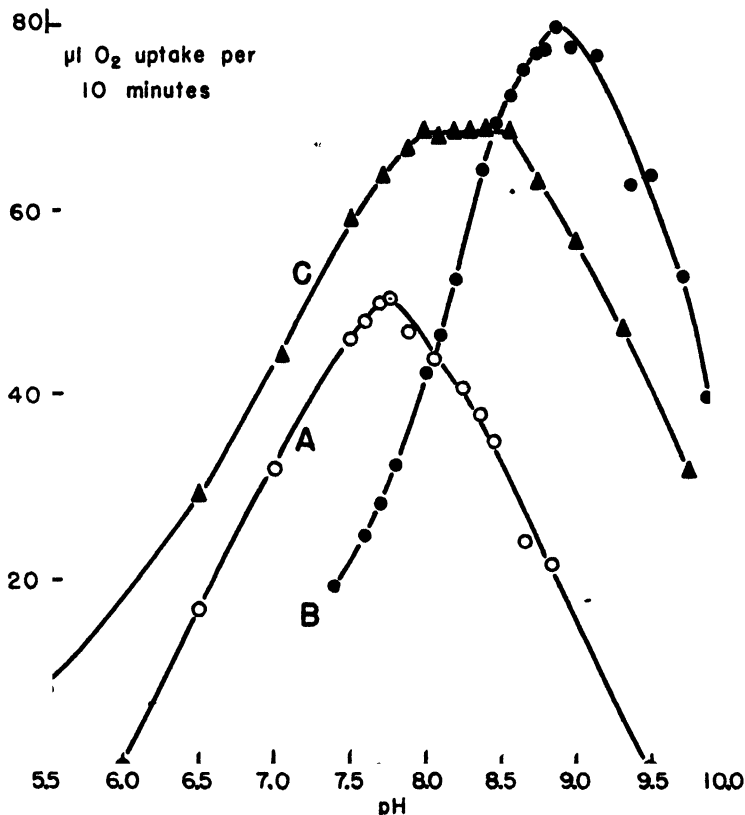


FIG. 2. Optimum pH for oxidation of glycolic and glyoxylic acids. The flasks contained Curve A, 0.5 ml. of 0.04 M glyoxylic acid; Curve B, 0.5 ml. of 0.02 M glycolic acid and 1.0 ml. of 0.3 M oxalic acid; Curve C, 0.5 ml. of 0.02 M glycolic acid. In addition each flask contained 1 ml. of phosphate-borate buffer, 0.1 ml. of salt-precipitated enzyme, and sufficient water to bring the total volume to 3.0 ml. The pH values are the average of the initial and final pH in the flasks after a 10 minute period of oxidation.

inhibits glyoxylic acid oxidation, the optimum pH for the oxidation of glycolic acid to glyoxylic acid can be determined in the presence of 0.1 M oxalic acid without confusion with the oxidation of glyoxylic acid. Under these conditions, glycolic acid is oxidized most rapidly at pH 8.9 (Curve B, Fig. 2). The pH-activity curve for complete oxidation of glycolic acid (Curve C, Fig. 2) is essentially a combination of Curves A and B.

**Ratios of Rates of Oxidation of L-Lactic, Glycolic, and Glyoxylic Acids—**

The ratios of the initial rates of oxidation of *l*-lactic acid and glycolic acid and of glyoxylic acid and glycolic acid are given in Table I for the various enzyme preparations. The results indicate reasonably constant ratios of activity on these substrates and suggest that only one enzyme is catalyzing the oxidation of *l*-lactic, glycolic, and glyoxylic acids.

TABLE I  
*Ratios of Rates of Oxidation of l-Lactic, Glycolic, and Glyoxylic Acids*

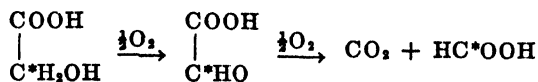
Enzyme preparation	Ratios of initial rates of oxidation	
	Lactic acid Glycolic acid	Glyoxylic acid Glycolic acid
Sap.....	0.50	0.39
Na <sub>2</sub> SO <sub>4</sub> ppt....	0.37	0.36
Salt ppt.....	0.35	0.38
“ “ pH 4.5 for 5 min . . .	0.34	0.24
Dialyzed . . . . .	0.40	0.26
Lyophilized . . . . .	0.34	0.34

#### DISCUSSION

The important position of pyruvic acid in carbohydrate metabolism lends interest to the investigation of the enzyme in plants which converts lactic to pyruvic acid. In contrast to the coenzyme-linked lactic acid dehydrogenase of muscle, the system oxidizing lactic acid in plants apparently has no cofactor requirement.

The function of glycolic acid in plants is unknown, although there are isolated reports of its occurrence in plants (8). Unpublished experiments in our laboratory on the separation of organic acids by partition chromatography have indicated the presence of glycolic acid in a number of plants. It may be present in sufficient concentration to support active respiration in a wide variety of plants. There are few references to the presence of glyoxylic acid in plants (9), and no function has been assigned to it nor to formic acid in plant metabolism.

The oxidation of a mole of glycolic acid by lyophilized enzyme preparations requires a mole of oxygen and produces a mole of CO<sub>2</sub>. The first step in the oxidation yields glyoxylic acid, and the oxidative decarboxylation of glyoxylic acid yields CO<sub>2</sub> and formic acid.



Tests with C<sup>14</sup>-labeled glycolic acid have shown that all the formic acid produced comes from the alcohol group and the CO<sub>2</sub> all comes from the carboxyl carbon. Furthermore, these tests have shown that probably no symmetrical molecule, *e.g.* oxalic acid, is an intermediate in the oxidation.



That formic acid and  $\text{CO}_2$  are the sole end-products of glycolic acid oxidation by the lyophilized enzyme is substantiated by data on respiration, by mercuric chloride analysis of the formic acid, by distribution of  $\text{C}^{14}$  after oxidation of labeled glycolic acid, by Duclaux values for formic acid, and by the isolation of *p*-bromophenacyl formate. Evidence that glyoxylic acid is the intermediate between glycolic and formic acids is based upon the isolation of the 2,4-dinitrophenylhydrazone of glyoxylic acid from an incompletely oxidized reaction mixture and upon the isolation of the semicarbazone of glyoxylic acid from reaction mixtures containing semicarbazide to prevent oxidation of glyoxylic acid.

The oxidation of glycolic acid to glyoxylic acid is analogous to the oxidation of *l*-lactic acid to pyruvic acid. When oxidation of glycolic acid is blocked at the glyoxylic acid stage, 0.5 mole of oxygen is taken up per mole of substrate and no  $\text{CO}_2$  is released. In the absence of inhibitors, glyoxylic acid is oxidized with the release of a mole of  $\text{CO}_2$  per mole of substrate and the utilization of 0.5 mole of oxygen.

The products of the oxidation of glycolic acid by tobacco sap or by crude salt-precipitated enzyme remain undetermined. The first reaction, as with the lyophilized enzyme, is the oxidation of glycolic acid to glyoxylic acid, but little  $\text{CO}_2$  and a correspondingly small amount of formic acid are produced as end-products. However, the uptake of oxygen remains 1 mole per mole of glycolic acid oxidized. Apparently, a reaction other than oxidation of glyoxylic acid to formic acid and  $\text{CO}_2$  is competing for the glyoxylic acid formed; such competition is absent in the lyophilized preparation. Oxalic acid has not been recovered even when very little  $\text{CO}_2$  was formed from the oxidation of glycolic acid by the crude enzyme preparations. *In vivo* studies with animals furnished labeled glycine by Sakami (10) and by Sprinson (11) indicate that glyoxylic acid and formic acid may be involved in the synthesis of serine and aspartic acid. The oxidation of glycolic acid and glyoxylic acid could serve as a source of formic acid. We have speculated earlier (1), inasmuch as the  $\alpha$ -hydroxy acid-oxidizing enzyme occurs only in the green parts of the plant, that glycolic acid itself or its oxidation products may be intermediates in the photosynthetic reactions. Although it was suggested (12) that the crude enzyme may oxidatively condense 2 molecules of glycolic acid to dihydroxymaleic acid and then oxidize this to yield 2 molecules of  $\text{CO}_2$  and a molecule of glyoxylic acid, no convincing experimental support for these reactions has been obtained to date.

#### SUMMARY

1. The enzyme from plants which oxidizes  $\alpha$ -hydroxy acids has been purified further by dialysis and lyophilization; when the dried material is redissolved in water, it has a  $Q_{O_2}$  (N) of about 50,000 on glycolic acid.

2. Tobacco sap, the salt-precipitated enzyme, and the lyophilized enzyme preparation each oxidized *l*-lactic acid to pyruvic acid.

3. Glycolic acid is oxidized by the lyophilized enzyme to formic acid and  $\text{CO}_2$ ; this requires a mole of oxygen per mole of substrate. The intermediate product is glyoxylic acid. The  $\text{CO}_2$  arises from the carboxyl carbon of glycolic acid and the formic acid from the alcohol group.

4. With tobacco sap or a crude enzyme preparation, 1 mole of oxygen is used per mole of glycolic acid oxidized, but little  $\text{CO}_2$  is produced. Glyoxylic acid is an intermediate product but the end-products have not been determined.

5. The optimum pH for the oxidation of glycolic to glyoxylic acid is 8.9, and for the oxidation of glyoxylic acid to formic acid the optimum is 7.7. The over-all oxidation of glycolic acid shows a nearly constant rate between pH 7.8 and 8.6.

6. The oxidation of glyoxylic acid is inhibited by high concentrations of oxalic acid and mesoxalic acid. In the presence of hydrazines, semicarbazide, or ethylenediamine, glyoxylic acid formed from glycolic acid is bound and undergoes no further oxidation. Ascorbic acid inhibits the oxidation of glycolic acid.

7. The ratios of initial rates of oxidation of (lactic acid)/(glycolic acid) and (glyoxylic acid)/(glycolic acid) by enzyme preparations treated in various ways indicate that one enzyme is oxidizing lactic, glycolic, and glyoxylic acids.

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# OBSERVATIONS ON THE INTERRELATION OF VITAMIN B<sub>12</sub>, FOLIC ACID, AND VITAMIN C IN THE CHICK\*

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It is well established that there is a definite relationship between folic acid, vitamin B<sub>12</sub>, and vitamin C. In the past two decades it has become apparent that vitamin C is related, directly or indirectly, to hematopoiesis (1). It has been shown that folic acid and vitamin C *in vivo* correct the abnormal tyrosine metabolism in the scorbutic guinea pig (2, 3).

Rodney, Swendseid, and Swanson (4) have shown that tyrosine oxidation in livers of folic acid-deficient rats, in which the deficiency was induced by sulfasuxidine, was significantly lower than that of a normal animal. They also showed that either liver extract, vitamin C, or folic acid would correct this abnormality *in vivo* but that only folic acid would correct it *in vitro*.

In 1944, Briggs *et al.* (5) observed a growth stimulation in chicks when vitamin C was added to a purified diet containing crude concentrates of folic acid. In 1946, it was shown that synthetic folic acid (6) and recently that vitamin B<sub>12</sub> (7) also stimulate growth, in the chick, on a semipurified ration.

It is the purpose of this paper to attempt to clarify, in part, the interrelationship of these three vitamins.

## EXPERIMENTAL

Straight run (New Hampshire ♂♂ × single comb white Leghorns ♀♀) cross-bred chicks, which were the progeny of hens fed Diet B-1

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for synthetic folic acid; to the Abbott Laboratories, North Chicago, Illinois, for haliver oil; and to E. I. du Pont de Nemours and Company, Inc., New Brunswick, New Jersey, for crystalline vitamin D<sub>3</sub>.

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described previously (8), were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The chicks were wing-banded and weighed at 1 day of age. Weights were recorded at weekly intervals.

All chicks were originally placed on a folic acid-deficient, purified ration containing sucrose 61 gm., alcohol-extracted casein 18 gm., gelatin 10 gm., Salts 5 (9) 6 gm., soy bean oil 5 gm., L-cystine 0.3 gm., thiamine hydrochloride 0.3 mg., riboflavin 0.6 mg., nicotinic acid 5.0 mg., pyridoxine hydrochloride 0.4 mg., calcium pantothenate 2.0 mg., choline chloride 150 mg., biotin 0.03 mg., inositol 100 mg., 2-methyl-1,4-naphthoquinone 0.05 mg., and  $\alpha$ -tocopherol 0.3 mg. Fortified haliver oil (60,000 U. S. P. units of vitamin A, 6000 U. S. P. units of vitamin D<sub>3</sub> per gm.) was given by dropper (2 drops per bird per week).

At the termination of the depletion period, which in all cases was 2 weeks, the chicks were sorted and divided into groups according to weight and per cent gain. A test period of 2 weeks followed, at the termination of which the birds were sacrificed, and the livers removed, frozen immediately, and stored at freezing temperatures until assayed. Individual livers were homogenated in a Waring blender with enough water to bring the concentration to 0.2 gm. per ml. Aliquots were then taken for both folic acid and vitamin B<sub>12</sub> determinations. Folic acid was extracted by autolysis of a 2.5 ml. aliquot of the homogenate in 10 ml. of a citrate-phosphate buffer at pH 4.5 for 18 to 20 hours at 37°. Autolysis at this pH has been shown to be satisfactory in obtaining maximum release in chick liver tissue (10, 11). Folic acid was measured microbiologically with *Streptococcus faecalis* R as a test organism with the medium of Luckey *et al.* (12).

Vitamin B<sub>12</sub> was extracted as follows: 2.5 ml. of liver homogenate were added to 10 ml. of 0.8 per cent NaHCO<sub>3</sub> solution, boiled for 5 minutes in a boiling water bath, and cooled, and 100 mg. of trypsin (Difco) were added to each sample. Toluene was added as a preservative and the samples were digested 24 to 30 hours at 37°. After incubation, the samples were neutralized, autoclaved 5 minutes at 120°, diluted, filtered, and assayed. Blanks were run with each extraction. It has been shown that this method will give maximum release of vitamin B<sub>12</sub> in chick liver tissue.<sup>1</sup> Vitamin B<sub>12</sub> was measured microbiologically with *Lactobacillus leichmannii* ATCC 4797 with the medium of Skeggs *et al.* (13), modified by the substitution of 10 ml. of fresh tomato juice per 100 ml. of double strength medium for the enzymatic digest of casein.

In all cases, the terms folic acid and vitamin B<sub>12</sub> will refer to *Streptococcus faecalis* R and *Lactobacillus leichmannii* activity respectively and

<sup>1</sup> Unpublished data from this laboratory.

will include those compounds that are active in promoting growth under the test conditions employed.

In Experiment 1, groups of twelve chicks each were used except in the groups receiving 200 and 500  $\gamma$  of folic acid per 100 gm. of ration in which ten birds were used per group. In Experiment 2 groups of twelve birds were used throughout. Crystalline vitamin B<sub>12</sub> was injected into the pectoral muscle with a 1 ml. calibrated syringe.

#### RESULTS AND DISCUSSION

The results are presented in Table I. The administration of vitamin C or vitamin B<sub>12</sub>, alone or together, gives a small growth response. Low levels of folic acid produce a greater response, but the addition of vitamin C and vitamin B<sub>12</sub>, or both, gives a further increase. In the series receiving 500  $\gamma$  of folic acid per 100 gm. of ration neither vitamin C nor vitamin B<sub>12</sub> produces any further response under the conditions of the experiment.

There appears to be a direct correlation between the level of folic acid stored in the liver and the rate of growth obtained. The addition of either vitamin C or vitamin B<sub>12</sub> with or without folic acid gives consistent if not significant increases in the level of folic acid stored in the liver. Administration of both vitamin C and vitamin B<sub>12</sub> raises the level of stored folic acid beyond that of either alone. When 500  $\gamma$  of folic acid per 100 gm. of ration are added to the basal ration, the addition of either vitamin C or vitamin B<sub>12</sub> has no effect on the level of stored folic acid.

As the level of folic acid is increased, the significance of increased folic acid stored in the liver induced by the addition of vitamin C, vitamin B<sub>12</sub>, or both, becomes less. This is in confirmation of the work of Moore *et al.* (14) who showed that the higher the intake of folic acid the more inefficient is its storage in the liver. This trend is followed by the growth data which show that the stimulation produced by vitamin B<sub>12</sub>, vitamin C, or both is relatively greater with low levels of folic acid and decreases as the folic acid level is increased. From such a comparison, it may be assumed that vitamin B<sub>12</sub> and vitamin C function, in part, by stimulating the synthesis of folic acid. Thus the administration of either vitamin B<sub>12</sub>, vitamin C, or both, is the equivalent of the addition of a higher level of folic acid. As further evidence, a comparison of Experiments 1 and 2 shows poorer growth in Experiment 2, but a more significant growth stimulation upon the addition of vitamin C, vitamin B<sub>12</sub>, or both. The folic acid stored in the liver follows the same trend. Further comparison of the folic acid stored in the livers of the groups receiving the unsupplemented basal ration shows that the chicks used in Experiment 2 were depleted of folic acid to a greater extent than those in Experiment 1. This accounts, in part, for the differences and again emphasizes the need for ex-

perimental animals from a controlled source; i.e., chicks which are the progeny of hens on a strictly controlled diet.

TABLE I

*Effect of Vitamin B<sub>12</sub>, Folic Acid, and Vitamin C on Chick Growth and Folic Acid and Vitamin B<sub>12</sub> Content of Livers*

Experiment No.	Supplement	Weight gain* during test	Vitamin B <sub>12</sub> per liver per bird†	Folic acid per liver per bird†
		gm.	γ	γ
1	None	19 (11)	0.05 ± 0.05	2.82 ± 0.58
	Vitamin B <sub>12</sub> (0.2 γ per day)†	40 (12)	0.12 ± 0.07	5.70 ± 2.76
	“ C (100 mg. %)	33 (12)	0.06 ± 0.05	4.65 ± 1.26
	“ B <sub>12</sub> (0.2 γ per day)† + vitamin C (100 mg. %)	59 (12)	0.24 ± 0.06	10.04 ± 4.15
	50 γ folic acid per 100 gm. ration	73 (11)	0.16 ± 0.04	4.03 ± 1.78
	50 “ “ “ 100 “ “ + vitamin B <sub>12</sub> (0.2 γ per day)†	108 (12)	0.28 ± 0.02	4.27 ± 2.48
	50 γ folic acid per 100 gm. ration + vitamin C (100 mg. %)	90 (11)	0.42 ± 0.18	6.58 ± 2.34
	50 γ folic acid per 100 gm. ration + vitamin B <sub>12</sub> (0.2 γ per day)† + vitamin C (100 mg. %)	121 (11)	0.61 ± 0.42	15.13 ± 4.81
	200 γ folic acid per 100 gm. ration	126 (10)	0.15 ± 0.06	15.60 ± 3.45
	200 γ “ “ “ 100 “ “ + vitamin B <sub>12</sub> (0.2 γ per day)†	131 (10)	0.15 ± 0.03	17.66 ± 2.44
	200 γ folic acid per 100 gm. ration + vitamin C (100 mg. %)	153 (10)	0.10 ± 0.03	20.70 ± 3.28
	200 γ folic acid per 100 gm. ration + vitamin B <sub>12</sub> (0.2 γ per day)† + vitamin C (100 mg. %)	150 (10)	0.18 ± 0.05	22.61 ± 4.49
	500 γ folic acid per 100 gm. ration	152 (10)	0.08 ± 0.02	14.05 ± 0.17
	500 “ “ “ “ 100 “ “ + vitamin B <sub>12</sub> (0.2 γ per day)†	147 (10)	0.16	17.50
	500 γ folic acid per 100 gm. ration + vitamin C (100 mg. %)	148 (10)	0.15	13.62
2	None	8 (7)	0.01 ± 0.00	1.20 ± 0.16
	Vitamin B <sub>12</sub> (0.1 γ per day)†	22 (10)	0.17 ± 0.07	2.96 ± 1.48
	“ C (100 mg. %)	14 (8)	0.05 ± 0.04	2.11 ± 0.85
	“ B <sub>12</sub> (0.1 γ per day)† + vitamin C (100 mg. %)	33 (11)	0.51 ± 0.12	4.44 ± 0.85
	Liver extract (0.5 U. S. P. units per day)†	87 (11)	0.58 ± 0.16	3.29 ± 0.95
	200 γ folic acid per 100 gm. ration	112 (12)	0.51 ± 0.53	6.87 ± 1.55
	200 “ “ “ “ 100 “ “ + vitamin B <sub>12</sub> (0.1 γ per day)†	134 (12)	0.52 ± 0.18	12.20 ± 2.12
	200 γ folic acid per 100 gm. ration + vitamin C (100 mg. %)	126 (12)	0.30 ± 0.11	11.73 ± 4.30

TABLE I—*Concluded*

Experiment No.	Supplement	Weight gain* during test	Vitamin B <sub>12</sub> per liver per bird†	Folic acid per liver per bird†
		gm.	γ	γ
	200 γ folic acid per 100 gm. ration + vitamin B <sub>12</sub> (0.1 γ per day)‡ + vitamin C (100 mg. %)	140 (12)	0.62 ± 0.06	21.35 ± 2.63
	500 γ folic acid per 100 gm. ration	140 (12)	0.31 ± 0.17	17.97 ± 5.47

\* The figures in parentheses represent the number of chicks surviving at the end of the test period.

† The values are the means of five individual livers and one pooled sample of remaining livers in Experiment 1 and seven individual livers and 1 pooled sample of remaining livers in Experiment 2.

‡ Injected.

Comparison of the groups receiving low levels of folic acid shows that the chick has the ability, although of uncertain efficiency, to store vitamin B<sub>12</sub>.

The ability of the chick to synthesize vitamin B<sub>12</sub> and to utilize the product is clearly shown. A comparison of the basal group in either experiment with groups receiving folic acid but no vitamin B<sub>12</sub> shows a significantly higher level of vitamin B<sub>12</sub> in the livers of the groups receiving folic acid and no vitamin B<sub>12</sub> than in groups receiving no folic acid and no vitamin B<sub>12</sub>. From these observations, it may be concluded that folic acid stimulates the synthesis of vitamin B<sub>12</sub>. The effect of vitamin C, either alone or with folic acid, on the synthesis of vitamin B<sub>12</sub> is doubtful.

That folic acid is synthesized by intestinal flora has been proved beyond any reasonable doubt. Even in the chick, which requires folic acid on a highly purified ration, the addition of 1 per cent sulfasuxidine to the diet increases the folic acid requirement 3-fold (6). It has been shown that vitamin C or other reducing agents stimulate the growth of certain microorganisms *in vitro* (15, 16). It is apparent that in these cases the action of vitamin C is that of an oxidation-reduction mechanism rather than that of a specific vitamin reaction.

The action of vitamin B<sub>12</sub> in stimulating the synthesis of folic acid is more obscure. It is improbable that this action is one of a specific vitamin function, but rather it is that of an indirect action. It is possible that this indirect action is also, in part, that of an oxidation-reduction mechanism, since it has been shown (17) that the cobalt in vitamin B<sub>12</sub> appears to be a six group coordination complex and that vitamin B<sub>12</sub> contains pyrrole-like compounds (18), suggesting a porphyrin nucleus.

## SUMMARY

Both vitamin C and vitamin B<sub>12</sub> stimulate growth in the chick when fed a semipurified ration with or without folic acid. The addition of both vitamin C and vitamin B<sub>12</sub> gives a greater growth response than either alone.

Both vitamin C and vitamin B<sub>12</sub> stimulate the synthesis of folic acid *in vivo*. The addition of both vitamin C and vitamin B<sub>12</sub> produces an increase of stored liver folic acid, which is in excess of that produced by either alone.

Folic acid stimulates the synthesis of vitamin B<sub>12</sub> in the chick, as measured by liver storage.

Possible mechanisms and interrelationships between folic acid, vitamin C, and vitamin B<sub>12</sub> have been discussed.

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## INDEX TO AUTHORS

### A

- Abrams, Adolph, Cohen, Philip P., and Meyer, Ovid O. The physical properties of a cryoglobulin obtained from lymph nodes and serum of a case of lymphosarcoma, 237
- Amberson, William R., Erdős, T., Chinn, Betty, and Ludes, Hans. Electrophoretic and ultracentrifugal analyses of protein extracted from whole mammalian muscles, 405
- Astwood, E. B., Greer, Monte A., and Ettlinger, Martin G. 1-5-Vinyl-2-thioxazolidone, an antithyroid compound from yellow turnip and from *Brassica* seeds, 121

### B

- Barber, Mary Alice, Basinski, Daniel H., and Mattill, H. A. Transamination in the muscles of animals deprived of vitamin E, 17
- Barker, H. A. See *Stadtman and Barker*, 221
- Barki, V. H., Feigelson, P., Collins, R. A., Hart, E. B., and Elvehjem, C. A. Factors influencing galactose utilization, 565
- Bartlett, Paul D., and Gaebler, Oliver H. Studies on the mechanism of nitrogen storage. II. Effects of anterior pituitary growth hormone preparations on kidney glutaminase, 523
- and —. III. The effects of anterior pituitary growth hormone preparations on the pyruvate-activated deamidation of glutamine in liver tissue, 529
- Basinski, Daniel H. See *Barber, Basinski, and Mattill*, 17
- Bass, Allan D., and Place, Elizabeth F. Uric acid and allantoin excretion in normal and tumor-bearing mice, 81
- Baumann, C. A. See *Sauberlich and Baumann*, 871
- Becker, Lillian. See *Evans and Bird*, 357
- Beinert, Helmut, and Reissmann, Kurt R. Studies on the incorporation of injected cytochrome *c* into tissue cells. I. Injection of non-radioactive cytochrome *c* into rats previously given radioiron, 367
- Benedict, Jean D., Forsham, Peter H., and Stetten, DeWitt, Jr. The metabolism of uric acid in the normal and gouty human studied with the aid of isotopic uric acid, 183
- Berg, Clarence P. See *Kallio and Berg*, 333
- Bernhart, F. W. See *Tomarelli, Norris, György, Hassinen, and Bernhart*, 879
- Bernheim, Frederick. See *Eadie, Bernheim, and Bernheim*, 449
- Bernheim, Mary L. C. See *Eadie, Bernheim, and Bernheim*, 449
- Binkley, Francis. Enzymatic hydrolysis and oxidation of monothiophosphate, 317
- Bird, Robert M. See *Evans and Bird*, 357
- Boas, Norman F. Isolation of hyaluronic acid from the cock's comb, 573
- Borek, Ernest. See *Prescott, Borek, Brecher, and Waelsch*, 273
- Brady, Roscoe O. See *Crandall, Brady, and Gurin*, 829
- Brecher, Arthur. See *Prescott, Borek, Brecher, and Waelsch*, 273
- Brown, Raymond A. See *Cann, Brown, and Kirkwood*, 161
- Burris, R. H. See *Tolbert, Clagett, and Burris*, 905

### C

- Cann, John R., Brown, Raymond A., and Kirkwood, John G. Application of electrophoresis-convection to the fractionation of bovine  $\gamma$ -globulin, 161

- Cerecedo, Leopold R. See *Soodak, Pircio, and Cerecedo*, 713
- Chinn, Betty. See *Amberson, Erdős, Chinn, and Ludes*, 405
- Clagett, C. O. See *Tolbert, Clagett, and Burris*, 905
- Clark, Harold W., Dounce, Alexander L., and Stotz, Elmer. An improved method for the extraction and purification of diphosphopyridine nucleotide, 459
- Cohen, Philip P. See *Abrams, Cohen, and Meyer*, 237
- See *Remmert and Cohen*, 431
- Collins, R. A. See *Barki, Feigelson, Collins, Hart, and Elvehjem*, 565
- Cook, Margaret. See *Lorber, Cook, and Meyer*, 475
- Cori, Carl F. See *Sutherland, Posier-nak, and Cori*, 153
- Corwin, Alsoph H. See *Freedman and Corwin*, 601
- Crandall, Dana I., Brady, Roscoe O., and Gurin, Samuel. Studies of acetoacetate formation with labeled carbon. II. The conversion of  $\gamma$ -(C<sub>1</sub>)-labeled octanoate to acetoacetate, 845
- and Gurin, Samuel. Studies of acetoacetate formation with labeled carbon. I. Experiments with pyruvate, acetate, and fatty acids in washed liver homogenates, 829
- D
- Day, Paul L. See *Dinning and Day*, 897
- Dekker, Charles A., Stone, David, and Fruton, Joseph S. A peptide from a marine alga, 719
- Deutsch, H. F. See *Fredericq and Deutsch*, 499
- Dietrich, L. S., Nichol, C. A., Monson, W. J., and Elvehjem, C. A. Observations on the interrelation of vitamin B<sub>12</sub>, folic acid, and vitamin C in the chick, 915
- Dinning, James S., and Day, Paul L. Creatinuria during recovery from aminopter-in-induced folic acid deficiency in the monkey, 897
- Dische, Zacharias. Spectrophotometric method for the determination of free pentose and pentose in nucleotides, 379
- Dornbush, A. C. See *Hoffmann, Stokstad, Hutchings, Dornbush, and Jukes*, 635
- Dounce, Alexander L. See *Clark, Dounce, and Stotz*, 459
- Dvornich, William, and Whistler, Roy L. Water-soluble polysaccharides of sweet corn, 889
- E
- Eadie, G. S., Bernheim, Frederick, and Bernheim, Mary L. C. The partial purification and properties of animal and plant hydantoins, 449
- Edwards, Sally. See *Richert, Edwards, and Westerfeld*, 255
- Eigen, Edward. See *Winsten and Eigen*, 109
- Elvehjem, C. A. See *Barki, Feigelson, Collins, Hart, and Elvehjem*, 565
- See *Dietrich, Nichol, Monson, and Elvehjem*, 915
- See *Williams and Elvehjem*, 559
- Erdős, T. See *Amberson, Erdős, Chinn, and Ludes*, 405
- Ettlinger, Martin G. See *Astwood, Greer, and Ettlinger*, 121
- Evans, John D., and Bird, Robert M. Metabolism of rabbit bone marrow *in vitro* in Ringer-bicarbonate medium containing no added glucose, 357
- F
- Feigelson, P. See *Barki, Feigelson, Collins, Hart, and Elvehjem*, 565
- Fodor, Paul J., and Greenstein, Jesse P. Separation of enzymatic activities toward chloroacetylalanine, chloroacetylalanylglycine, and glycylalanine, 549
- Foote, Murray W., Little, John E., and Sproston, Thomas J. On naphthoquinones as the inhibitors of spore germination of fungi, 481

- Forsham, Peter H. See *Benedict, Forsham, and Stetten*, 183  
 Fredericq, Eugene, and Deutsch, H. F. Studies on ovomucoid, 499  
 Freedman, Leon D., and Corwin, Al-soph H. Oxidation-reduction potentials of thiol-disulfide systems, 601  
 Friedman, Bernice. See *Weinhouse, Millington, and Friedman*, 489  
 Fruton, Joseph S. See *Dekker, Stone, and Fruton*, 719

## G

- Gaebler, Oliver H. See *Bartlett and Gaebler*, 523, 529  
 Gilbert, James B. See *Price, Meister, Gilbert, and Greenstein*, 535  
 Gilmore, Richard C., Jr., and Samuels, Leo T. The effect of previous diet on the metabolic activity of the isolated rat diaphragm, 813  
 Goldzieher, Joseph W. See *Stone and Goldzieher*, 511  
 Greco, Antoinette E. See *Maver and Greco*, 853, 861  
 Greenberg, G. Robert. Inhibitory effect of muscle adenylic acid on anaerobic glycolysis of brain, 781  
 Greenstein, Jesse P. See *Fodor and Greenstein*, 549  
 —. See *Price, Meister, Gilbert, and Greenstein*, 535  
 Greer, Monte A. See *Astwood, Greer, and Ettlinger*, 121  
 Grunert, R. R., and Phillips, Paul H. Sodium and its relation to alloxan diabetes and glutathione, 821  
 Gunsalus, I. C. See *Wood and Gunsalus*, 171  
 Gurin, Samuel. See *Crandall, Brady, and Gurin*, 845  
 —. See *Crandall and Gurin*, 829  
 György, Paul. See *Tomarelli, Norris, György, Hassinen, and Bernhart*, 879
- H  
 Hassinen, J. B. See *Tomarelli, Norris, György, Hassinen, and Bernhart*, 879  
 Hastings, A. Baird. See *Villee and Hastings*, 131  
 Heath, Arthur E. See *Jones, Koch, Heath, and Munson*, 755  
 Henderson, L. M. Quinolinic acid metabolism. II. Replacement of nicotinic acid for the growth of the rat and *Neurospora*, 677  
 — and Hirsch, Herbert M. Quinolinic acid metabolism. I. Urinary excretion by the rat following tryptophan and 3-hydroxyanthranilic acid administration, 667  
 — and Ramasarma, G. B. Quinolinic acid metabolism. III. Formation from 3-hydroxyanthranilic acid by rat liver preparations, 687  
 —, Ramasarma, G. B., and Johnson, B. Connor. Quinolinic acid metabolism. IV. Urinary excretion by man and other mammals as affected by the ingestion of tryptophan, 731  
 Herbst, Edward J., and Snell, Esmond E. Putrescine and related compounds as growth factors for *Hemophilus parainfluenzae*, 47  
 Hess, W. C. The rates of absorption of and the formation of liver glycogen by methionine, cystine, and cysteine, 23  
 Hirsch, Herbert M. See *Henderson and Hirsch*, 667  
 Hixon, Walter S. See *Hunter and Hixon*, 67, 73  
 Hoch, Frederic L., and Vallee, Bert L. Precipitation by trichloroacetic acid as a simplification in the determination of zinc in blood and its components, 295  
 Hoffmann, C. E., Stokstad, E. L. R., Hutchings, B. L., Dornbush, A. C., and Jukes, Thomas H. The microbiological assay of vitamin B<sub>12</sub> with *Lactobacillus leichmannii*, 635  
 Hundley, James M. Influence of fructose and other carbohydrates on the niacin requirement of the rat, 1

- Hunter, F. Edmund, Jr., and Hixon, Walter S. Anaerobic phosphorylation due to the dismutation of  $\alpha$ -ketoglutaric acid in the presence of ammonia, 67  
 —, and —. Phosphorylation coupled with the oxidation of  $\alpha$ -ketoglutaric acid, 73  
 Hutchings, B. L. See *Hoffmann, Stokstad, Hutchings, Dornbush, and Jukes*, 635

## J

- Jacobs, Walter A., and Sato, Yoshio. The veratrine alkaloids. XXX. A further study of the structure of veratramine and jervine, 55  
 Johnson, B. Connor. See *Henderson, Ramasarma, and Johnson*, 731  
 Johnson, Marvin J. A rapid micro-method for estimation of non-volatile organic matter, 707  
 —. See *Park and Johnson*, 149  
 Jones, Mary Ellen, Koch, F. C., Heath, Arthur E., and Munson, Paul L. Isolation of  $\alpha$ -menopalmitin from hog pancreas, 755  
 Jukes, Thomas H. See *Hoffmann, Stokstad, Hutchings, Dornbush, and Jukes*, 635

## K

- Kallio, Reino E., and Berg, Clarence P. Tryptophan metabolism. XII. Tryptophan, kynurenine, and related compounds as precursors of nicotinic acid, 333  
 Kaufman, Seymour, and Neurath, Hans. Structural requirements of specific inhibitors for  $\alpha$ -chymotrypsin, 623  
 Kerr, Stanley E., Seraldarian, Krikor, and Wargon, Maria. Studies on ribonucleic acid. II. Methods of analysis, 761  
 —, —, and —. III. On the composition of the ribonucleic acid of beef pancreas, with notes on the action of ribonuclease, 773  
 Kirkwood, John G. See *Cann, Brown, and Kirkwood*, 161

- Koch, F. C. See *Jones, Koch, Heath, and Munson*, 755  
 Kozloff, Lloyd M., and Putnam, Frank W. Biochemical studies of virus reproduction. II. Chemical composition of *Escherichia coli* bacteriophage T<sub>1</sub> and its host, 207  
 Krahl, M. E. See *Park and Krahl*, 247

## L

- Lampson, George P., and Lardy, Henry A. Phosphoric esters of biological importance. II. The synthesis of glucose-6-phosphate from 1,2-isopropylidene-5,6-anhydro-D-glucufuranose, 693  
 — and —. III. The synthesis of propanediol phosphate, 697  
 Lardy, Henry A. See *Lampson and Lardy*, 693, 697  
 —. See *Totton and Lardy*, 701  
 Layton, William M., Jr. See *Zimmerman and Layton*, 141  
 Lehninger, Albert L., and Smith, Sylvia Wagner. Efficiency of phosphorylation coupled to electron transport between dihydrodiphosphopyridine nucleotide and oxygen, 415  
 Lerner, Aaron Bunsen. On the metabolism of phenylalanine and tyrosine, 281  
 Lerner, Edwin M., and Mueller, J. Howard. The rôle of glutamine in the glucose metabolism of *Clostridium tetani*, 43  
 Little, John E. See *Foote, Little, and Sproston*, 481  
 Lorber, Victor, Cook, Margaret, and Meyer, Joe. Conversion of octanoic acid to rat liver glycogen, studied with C<sup>14</sup>, C<sup>13</sup>-labeled octanoate, 475  
 Ludes, Hans. See *Amberson, Erdős, Chinn, and Ludes*, 405

## M

- MacLeod, Leslie D. Determination of alcohol by microdiffusion, 323  
 Marquette, Mona M. See *Schweigert and Marquette*, 199  
 Martindale, William E. See *Dinning and Day*, 897

- Mason, Howard S. The chemistry of melanin. VI. Mechanism of the oxidation of catechol by tyrosinase, 803
- Mattill, H. A. See *Barber, Basinski, and Mattill*, 17
- Maver, Mary E., and Greco, Antoinette E. The hydrolysis of nucleoproteins by cathepsins from calf thymus, 853  
— and —. The nuclease activities of cathepsin preparations from calf spleen and thymus, 861
- Meister, Alton. See *Price, Meister, Gilbert, and Greenstein*, 535
- Meyer, Joe. See *Lorber, Cook, and Meyer*, 475
- Meyer, Ovid O. See *Abrams, Cohen, and Meyer*, 237
- Miller, Pauline A. See *Mueller and Miller*, 39
- Millington, Ruth H. See *Weinhouse and Millington*, 645  
— See *Weinhouse, Millington, and Friedman*, 489
- Monson, W. J. See *Dietrich, Nichol, Monson, and Elvehjem*, 915
- Mueller, J. Howard, and Miller, Pauline A. Glutamine in the production of tetanus toxin, 39  
— See *Lerner and Mueller*, 43
- Munson, Paul L. See *Jones, Koch, Heath, and Munson*, 755

## N

- Nachlas, Marvin M., and Seligman, Arnold M. Evidence for the specificity of esterase and lipase by the use of three chromogenic substrates, 343
- Neurath, Hans. See *Kaufman and Neurath*, 623  
— See *Snook and Neurath*, 789
- Nichol, C. A. See *Dietrich, Nichol, Monson, and Elvehjem*, 915
- Norris, R. F. See *Tomarelli, Norris, György, Hassinen, and Bernhart*, 879

## O

- Olds, Claire Jeanne. See *Dinning and Day*, 897

## P

- Pankey, Margaret J. See *Dinning and Day*, 897
- Pardee, Arthur B., and Potter, Van R. Factors affecting the maintenance of oxidative phosphorylation in a kidney homogenate system, 739
- Park, C. R., and KrahI, M. E. Effect of pituitary extracts upon glucose uptake by diaphragms from normal, hypophysectomized, and hypophysectomized-adrenalectomized rats, 247
- Park, James T., and Johnson, Marvin J. A submicrodetermination of glucose, 149
- Pearson, Harold E., and Winzler, Richard J. Oxidative and glycolytic metabolism of minced day-old mouse brain in relation to propagation of Theiler's GD VII virus, 577  
— See *Rafelson, Winzler, and Pearson*, 583, 595
- Peterson, D. H., and Reineke, L. M. The chemistry of circulin; chromatographic isolation of the amino acid constituents with powdered cellulose, 95
- Phillips, Paul H. See *Grunert and Phillips*, 821
- Pircio, Anthony. See *Soodak, Pircio, and Cerecedo*, 713
- Place, Elizabeth F. See *Bass and Place*, 81
- Posternak, Theodore. See *Sutherland, Posternak, and Cori*, 153
- Potter, Van R. See *Pardee and Potter*, 739
- Prescott, Blanche A., Borek, Ernest, Brecher, Arthur, and Walsch, Heinrich. Studies on oligophrenia phenylpyruvica. I. Microbiological determination of L- and D-phenylalanine and of phenyllactic acid, 273
- Price, Vincent E., Meister, Alton, Gilbert, James B., and Greenstein, Jesse P. The separation of dehydropeptidase and analogous L- and D-peptidases, 535
- Putnam, Frank W. See *Kozloff and Putnam*, 207

## R

- Rafelson, Max E., Jr., Winzler, Richard J., and Pearson, Harold E.** The effects of Theiler's GD VII virus on P<sup>32</sup> uptake by minced one day-old mouse brain, 583
- , —, and —. The effects of Theiler's GD VII virus on the incorporation of radioactive carbon from glucose into minced one day-old mouse brain, 595
- Ramasarma, G. B.** See *Henderson and Ramasarma*, 687
- , —. See *Henderson, Ramasarma, and Johnson*, 731
- Reineke, L. M.** See *Peterson and Reineke*, 95
- Reissmann, Kurt R.** See *Beinert and Reissmann*, 367
- Remmert, LeMar F., and Cohen, Philip P.** Partial purification and properties of a proteolytic enzyme of human serum, 431
- Richert, Dan A., Edwards, Sally, and Westerfeld, W. W.** On the determination of liver xanthine oxidase and the respiration of rat liver homogenates, 255
- Roderuck, Charlotte E.** Analysis of certain components of skeletal muscle during vitamin E deficiency, 11
- Rose, William C., Smith, Leonard C., Womack, Madelyn, and Shane, Morton.** The utilization of the nitrogen of ammonium salts, urea, and certain other compounds in the synthesis of non-essential amino acids *in vivo*, 307

## S

- Sacks, Jacob.** A fractionation procedure for the acid-soluble phosphorus compounds of liver, 655
- Samuels, Leo T.** See *Gilmore and Samuels*, 813
- Sato, Yoshio.** See *Jacobs and Sato*, 55
- Sauberlich, H. E.** The effect of folic acid upon the urinary excretion of the growth factor required by *Leuconostoc citrovorum*, 467
- Sauberlich, H. E., and Baumann, C. A.** Further studies on the factor required by *Leuconostoc citrovorum* 8081, 871
- Schweigert, B. S., and Marquette, Mona M.** Further studies on the metabolism of 3-hydroxyanthranilic acid by rat liver slices and homogenates, 199
- Sealock, Robert Ridgely, and White, Hilda S.** Neopyrithiamine and the thiaminase of fish tissues, 393
- Selp, Muriel.** See *Evans and Bird*, 357
- Seligman, Arnold M.** See *Nachlas and Seligman*, 343
- Seraidarian, Krikor.** See *Kerr, Seridarian, and Wargon*, 761, 773
- Shane, Morton.** See *Rose, Smith, Womack, and Shane*, 307
- Shaw, Elliott, and Woolley, D. W.** A new and convenient synthesis of 4-amino-5-imidazolecarboxamide, 89
- Smith, Leonard C.** See *Rose, Smith, Womack, and Shane*, 307
- Smith, Sylvia Wagner.** See *Lehninger and Smith*, 415
- Snell, Esmond E.** See *Herbst and Snell*, 47
- Snoke, John E., and Neurath, Hans.** Structural requirements of specific substrates for carboxypeptidase, 789
- Soodak, Morris, Pircio, Anthony, and Cerecedo, Leopold R.** A colorimetric method for the estimation of uracil and cytosine, 713
- Sproston, Thomas J.** See *Foote, Little, and Sproston*, 481
- Stadtman, E. R., and Barker, H. A.** Fatty acid synthesis by enzyme preparations of *Clostridium kluyveri*. V. A consideration of postulated 4-carbon intermediates in butyrate synthesis, 221
- Stetten, DeWitt, Jr.** See *Benedict, Forsham, and Stetten*, 183
- Stetten, Marjorie R.** Some aspects of the metabolism of hydroxyproline, studied with the aid of isotopic nitrogen, 31

- Stokstad, E. L. R. See *Hofmann, Stokstad, Hutchings, Dornbush, and Jukes*, 635
- Stone, David. See *Dekker, Stone, and Fruton*, 719
- Stone, Gilbert C. H., and Goldzieher, Joseph W. A rapid colorimetric method for the determination of sodium in biological fluids and particularly in serum, 511
- Stotz, Elmer. See *Clark, Dounce, and Stotz*, 459
- Sutherland, Earl W., Posternak, Theodore, and Cori, Carl F. Mechanism of the phosphoglyceric mutase reaction, 153

## T

- Taussky, Hertha H. A microcolorimetric method for the determination of citric acid. II. A note on the substitution of ferrous sulfate for hydrazine sulfate as the reducing agent, 195
- Tolbert, N. E., Clagett, C. O., and Burris, R. H. Products of the oxidation of glycolic acid and *l*-lactic acid by enzymes from tobacco leaves, 905
- Tomarelli, Rudolph M., Norris, R. F., György, Paul, Hassinen, J. B., and Bernhart, F. W. The nutrition of variants of *Lactobacillus bifidus*, 879
- Totton, Ezra L., and Lardy, Henry A. Phosphoric esters of biological importance. IV. The synthesis and biological activity of D-tagatose-6-phosphate, 701

## V

- Vallee, Bert L. See *Hoch and Vallee*, 295
- Villee, Claude A., and Hastings, A. Baird. The utilization *in vitro* of C<sup>14</sup>-labeled acetate and pyruvate by diaphragm muscle of rat, 131

## W

- Waelisch, Heinrich. See *Prescott, Borek, Brecher, and Waelisch*, 273
- Wargon, Maria. See *Kerr, Seraidarian, and Wargon*, 761, 773
- Weinhouse, Sidney, and Millington, Ruth H. Ketone body formation from tyrosine, 645
- , —, and Friedman, Bernice. The effect of carbohydrate on the oxidation of fatty acids by liver slices, 489
- Wells, Dolph T. See *Dining and Day*, 897
- Westerfeld, W. W. See *Richert, Edwards, and Westerfeld*, 255
- Whistler, Roy L. See *Dvonch and Whistler*, 889
- White, Hilda S. See *Sealock and White*, 393
- Williams, J. N., Jr., and Elvehjem, C. A. The relation of amino acid availability in dietary protein to liver enzyme activity, 559
- Winsten, Walter A., and Eigen, Edward. Paper chromatography of vitamin B<sub>12</sub> and related bacterial growth factors, 109
- Winzler, Richard J. See *Pearson and Winzler*, 577
- , See *Rafelson, Winzler, and Pearson*, 583, 595
- Womack, Madelyn. See *Rose, Smith, Womack, and Shane*, 307
- Wood, W. A., and Gunsalus, I. C. Serine and threonine deaminases of *Escherichia coli*: activators for a cell-free enzyme, 171
- Woolley, D. W. See *Shaw and Woolley*, 89

## Z

- Zimmerman, Walter J., and Layton, William M., Jr. A polarographic micromethod for the determination of blood chloride, 141





## INDEX TO SUBJECTS

### A

- Acetate:** Aceto-. *See* Acetoacetate
- Acetoacetate formation, liver, *Crandall and Gurin*, 829
- Carbon<sup>14</sup>-labeled, diaphragm, utilization, *Villee and Hastings*, 121
- Acetoacetate:** Formation, labeled carbon in study, *Crandall and Gurin*, 829  
*Crandall, Brady, and Gurin*, 845
- Octanoate,  $\gamma$ -(C<sub>7</sub>)-labeled, conversion from, *Crandall, Brady, and Gurin*, 845
- Adenylic acid:** Muscle, brain glycolysis, effect, *Greenberg*, 781
- Adrenalectomy-hypophysectomy:** Diaphragm glucose uptake, pituitary extract effect, *Park and Krahl*, 247
- Alanine:** Chloroacetyl-. *See* Chloroacetylalanine
- Glycyl-. *See* Glycylalanine
- Phenyl-. *See* Phenylalanine
- Alcohol:** Determination, microdiffusion, *MacLeod*, 323
- Alga:** Marine, peptide, *Dekker, Stone, and Fruton*, 719
- Alkaloid(s):** Veratrine, *Jacobs and Sato*, 55
- Allantoin:** Excretion, tumor-bearing mice, *Bass and Place*, 81
- Alloxan:** Diabetes, sodium relation, *Grunert and Phillips*, 821
- Amide:** Amino-5-imidazolecarbox-. *See* Amino-5-imidazolecarboxamide
- Amino acid(s):** Circulin, isolation, chromatographic, *Peterson and Reineke*, 95
- Protein, liver enzymes, relation, *Williams and Elvehjem*, 559
- Synthesis *in vivo*, ammonium salts and urea nitrogen, utilization, *Rose, Smith, Womack, and Shane*, 307
- Amino-5-imidazolecarboxamide:** 4-, synthesis, *Shaw and Woolley*, 89
- Aminopterin:** -Induced folic acid deficiency, creatinuria, monkey, *Dinning and Day*, 897

- Ammonium salt(s):** Nitrogen, amino acid synthesis *in vivo*, *Rose, Smith, Womack, and Shane*, 307
- Anthranilic acid:** 3-Hydroxy-, metabolism, liver, *Schweigert and Marquette*, 199
- , quinolinic acid excretion, effect, *Henderson and Hirsch*, 667
- , — — formation, liver, *Henderson and Ramasarma*, 687

### B

- Bacillus:** *See also* *Lactobacillus*
- Bacteria:** Growth factors, chromatography, *Winsten and Eigen*, 109
- See also* *Clostridium, Escherichia, Hemophilus, Leuconostoc*
- Bacteriophage:** T<sub>1</sub>, *Escherichia coli*, chemical composition, *Kozloff and Putnam*, 207
- Blood:** Chloride, determination, micro-, polarographic, *Zimmerman and Layton*, 141
- Zinc determination, trichloroacetic acid use, *Hoch and Vallee*, 295
- Blood serum:** Cryoglobulin, lymphosarcoma, physical properties, *Abrams, Cohen, and Meyer*, 237
- Enzyme, proteolytic, purification and properties, *Remmert and Cohen*, 431
- Sodium, determination, colorimetric, *Stone and Goldzieher*, 511
- Bone marrow:** Metabolism, *Evans and Bird*, 357
- Brain:** Glycolysis, muscle adenylic acid, effect, *Greenberg*, 781
- Metabolism, Theiler's GD VII virus, relation, *Pearson and Winzler*, 577
- Phosphorus<sup>32</sup> uptake, Theiler's GD VII virus, effect, *Rafelson, Winzler, and Pearson*, 583
- Radioactive carbon from glucose incorporation, Theiler's GD VII virus, effect, *Rafelson, Winzler, and Pearson*, 595

- Brassica:** *l*-5-Vinyl-2-thiooxazolidone, thyroid, effect, *Astwood, Greer, and Ettlinger*, 121
- Butyrate:** Synthesis, 4-carbon intermediates, *Clostridium kluyveri* enzyme preparations, *Stadtman and Barker*, 221
- C**
- Carbohydrate(s):** Fatty acids, oxidation, liver, effect, *Weinhouse, Milington, and Friedman*, 489  
Niacin requirement, effect, *Hundley*, 1
- Carbon:** Labeled, acetoacetate formation, use in study, *Crandall and Gurin*, 829  
*Crandall, Brady, and Gurin*, 845  
Mass 14-labeled acetate, diaphragm, utilization, *Villee and Hastings*, 131  
— pyruvate, diaphragm, utilization, *Villee and Hastings*, 131  
Radioactive, in glucose, brain incorporation, Theiler's GD VII virus, effect, *Rafelson, Winzler, and Pearson*, 595
- Carboxypeptidase.** Substrate, structural requirements, *Snoke and Neurath*, 789
- Catechol:** Oxidation by tyrosinase, mechanism, *Mason*, 803
- Cathepsin(s):** Spleen, nuclease, *Maver and Greco*, 861  
Thymus, nuclease, *Maver and Greco*, 861  
— nucleoproteins, hydrolysis, *Maver and Greco*, 853
- Chick:** Vitamin B<sub>12</sub>, folic acid, and vitamin C, interrelation, *Dietrich, Nichol, Monson, and Elvehjem*, 915
- Chloride:** Blood, determination, micro-polarographic, *Zimmerman and Layton*, 141
- Chloroacetylalanine:** Enzyme effect, *Fodor and Greenstein*, 549
- Chloroacetylalanylglycine:** Enzyme effect, *Fodor and Greenstein*, 549
- Chymotrypsin:**  $\alpha$ -, inhibitors, *Kaufman and Neurath*, 623
- Circulin:** Amino acids, isolation, chromatographic, *Peterson and Reineke*, 95
- Citric acid:** Determination, microcolorimetric, *Taussky*, 195
- Clostridium kluyveri:** Enzyme preparations, butyrate synthesis, 4-carbon intermediates, *Stadtman and Barker*, 221  
— —, fatty acid synthesis, *Stadtman and Barker*, 221
- Clostridium tetani:** Glucose metabolism, glutamine rôle, *Lerner and Mueller*, 43
- Cock:** Comb, hyaluronic acid isolation, *Boas*, 573
- Corn:** Sweet, polysaccharides, water-soluble, *Dvornch and Whistler*, 889
- Creatinuria:** Folic acid deficiency, aminopterin-induced, monkey, *Dinning and Day*, 897
- Cryoglobulin:** Lymph nodes and blood serum, lymphosarcoma, physical properties, *Abrams, Cohen, and Meyer*, 237
- Cysteine:** Liver glycogen formation, effect, *Hess*, 23
- Cystine:** Liver glycogen formation, effect, *Hess*, 23
- Cytochrome:** *c*, non-radioactive, injection effect, *Beinert and Reissmann*, 367  
—, tissue cells, incorporation, *Beinert and Reissmann*, 367
- Cytosine:** Determination, colorimetric, *Soodak, Pircio, and Cerecedo*, 713
- D**
- Deaminase:** Serine and threonine, *Escherichia coli*, *Wood and Gunsalus*, 171
- Dehydropeptidase:** L- and D-peptidases and, separation, *Price, Meister, Gilbert, and Greenstein*, 535
- Diabetes:** Alloxan, sodium relation, *Grunert and Phillips*, 821
- Diaphragm:** Carbon<sup>14</sup>-labeled acetate and pyruvate utilization, *Villee and Hastings*, 131

**Diaphragm—continued:**

- Glucose uptake, pituitary extract effect, *Park and Krahl*, 247  
 Metabolism, diet effect, *Gilmore and Samuels*, 813  
**Diet:** Diaphragm metabolism, effect, *Gilmore and Samuels*, 813  
**Dihydrodiphosphopyridine nucleotide:** Phosphorylation, oxygen and, *Lehninger and Smith*, 415  
**Diphosphopyridine nucleotide:** Extraction and purification, *Clark, Dounce, and Stots*, 459  
**Disulfide:** Thiol-, systems, oxidation-reduction potentials, *Freedman and Corwin*, 601

**E**

- Enzyme(s):** Chloroacetylalanine, effect, *Fodor and Greenstein*, 549  
 Chloroacetylalanylglycine, effect, *Fodor and Greenstein*, 549  
*Clostridium kluveri*, butyrate synthesis, 4-carbon intermediates, *Stadtman and Barker*, 221  
 — —, fatty acid synthesis, *Stadtman and Barker*, 221  
 Glycylalanine, effect, *Fodor and Greenstein*, 549  
 Liver, protein amino acids, relation, *Williams and Elvehjem*, 559  
 Monothiophosphate, hydrolysis and oxidation, *Binkley*, 317  
 Proteolysis, blood serum, purification and properties, *Remmert and Cohen*, 431  
 Tobacco leaf, glycolic acid and L-lactic acid, oxidation products, *Tolbert, Clagett, and Burris*, 905  
*See also Carboxypeptidase, Chymotrypsin, etc.*  
**Escherichia coli:** Bacteriophage T<sub>2</sub> and host, chemical composition, *Kozloff and Putnam*, 207  
 Serine and threonine deaminases, *Wood and Gunsalus*, 171  
**Esterase:** Specificity, *Nachlas and Seligman*, 343

**F**

- Fatty acid(s):** Acetoacetate formation, liver, *Crandall and Gurin*, 829  
 Oxidation, liver, carbohydrates, effect, *Weinhouse, Millington, and Friedman*, 489  
 Synthesis, *Clostridium kluveri* enzyme preparations, *Stadtman and Barker*, 221  
**Fish:** Neopyrithiamine and thiaminase, relation, *Sealock and White*, 393  
**Folic acid:** Deficiency, aminopterin-induced, creatinuria, monkey, *Dinning and Day*, 897  
 Urine, *Leuconostoc citrovorum* growth factor, effect, *Sauberlich*, 467  
 Vitamin B<sub>12</sub> and vitamin C, interrelation, chick, *Dietrich, Nichol, Monson, and Elvehjem*, 915  
**Fructose:** Niacin requirement, effect, *Hundley*, 1  
**Fungus:** Germination, naphthoquinones, effect, *Foote, Little, and Sproston*, 481  
**Furanose:** 1,2-Isopropylidene-5,6-anhydro-D-glucosyl, glucose-6-phosphate synthesis from, *Lampson and Lardy*, 693

**G**

- Galactose:** Utilization, factors influencing, *Barki, Feigelson, Collins, Hart, and Elvehjem*, 565  
**Globulin(s):** Cryo-. *See Cryoglobulin*  
 γ-, fractionation, electrophoresis-convection use, *Cann, Brown, and Kirkwood*, 161  
**Glucose:** Determination, submicro-, *Park and Johnson*, 149  
 Metabolism, *Clostridium tetani*, glutamine rôle, *Lerner and Mueller*, 43  
 Radioactive carbon-containing, brain incorporation, Theiler's GD VII virus, effect, *Rafelson, Winsler, and Pearson*, 595  
 Uptake, diaphragm, pituitary extract effect, *Park and Krahl*, 247

**Glucose-6-phosphate:** Synthesis from 1,2 - isopropylidene - 5,6 - anhydro-D-glucofuranose, *Lampson and Lardy*, 693

**Glutaminase:** Kidney, anterior pituitary growth hormone, effect, *Bartlett and Gaebler*, 523

**Glutamine:** *Clostridium tetani*, glucose metabolism, rôle, *Lerner and Mueller*, 43  
Liver, deamidation, anterior pituitary growth hormone, effect, *Bartlett and Gaebler*, 529  
Tetanus toxin, production, effect, *Mueller and Miller*, 39

**Glutaric acid:**  $\alpha$ -Keto-, dismutation, anaerobic phosphorylation, *Hunter and Hixon*, 67  
—, oxidation and phosphorylation, *Hunter and Hixon*, 73

**Glutathione:** Sodium relation, *Grunert and Phillips*, 821

**Glycine:** Chloroacetylalanyl-, enzyme effect, *Fodor and Greenstein*, 549

**Glycogen:** Liver, formation, methionine, cystine, and cysteine effect, *Hess*, 23  
—, octanoic acid conversion to,  $C^{14}$ ,  $C^{12}$ -labeled octanoate use in study, *Lorber, Cook, and Meyer*, 475

**Glycolic acid:** Oxidation, tobacco leaf enzymes, *Tolbert, Clagett, and Burris*, 905

**Glycolysis:** Brain, muscle adenylic acid, effect, *Greenberg*, 781  
—, Theiler's GD VII virus, relation, *Pearson and Winzler*, 577

**Glycylalanine:** Enzyme effect, *Fodor and Greenstein*, 549

**Gout:** Uric acid metabolism, isotopic uric acid in study, *Benedict, Forsham, and Stetten*, 183

**Growth:** Hormone, anterior pituitary, kidney glutaminase, effect, *Bartlett and Gaebler*, 523  
—, —, liver glutamine, deamidation, effect, *Bartlett and Gaebler*, 529  
Nicotinic acid replacement, effect, *Henderson*, 677

## H

**Hemophilus parainfluenzae:** Growth, putrescine and related compounds, effect, *Herbst and Snell*, 47

**Hyaluronic acid:** Cock comb, isolation, *Boas*, 573

**Hydantoinase(s):** Purification and properties, *Eadie, Bernheim, and Bernheim*, 449

**Hydroxyanthranilic acid:** 3-, metabolism, liver, *Schweigert and Marquette*, 199  
—, quinolinic acid excretion, effect, *Henderson and Hirsch*, 667  
—, — — formation from, liver, *Henderson and Ramasarma*, 687

**Hydroxyproline:** Metabolism, isotopic nitrogen in study, *Stetten*, 31

**Hypophysectomy:** Diaphragm glucose uptake, pituitary extract effect, *Park and Krahl*, 247

**Hypophysectomy-adrenalectomy:** Diaphragm glucose uptake, pituitary extract effect, *Park and Krahl*, 247

## I

**Isopropylidene - 5,6-anhydro-D-glucofuranose:** 1,2-, glucose-6-phosphate synthesis from, *Lampson and Lardy*, 693

## J

**Jervine:** Chemical constitution, *Jacobs and Sato*, 55

## K

**Ketoglutaric acid:**  $\alpha$ -, dismutation, anaerobic phosphorylation, *Hunter and Hixon*, 67  
—, oxidation and phosphorylation, *Hunter and Hixon*, 73

**Ketone bodies:** Formation from tyrosine, *Weinhouse and Millington*, 645

**Kidney:** Glutaminase, anterior pituitary growth hormone, effect, *Bartlett and Gaebler*, 523  
Phosphorylation, oxidative, factors affecting, *Pardee and Potter*, 739

**Kynurenine:** Nicotinic acid precursor, *Kallio and Berg*, 333

## L

- Lactic acid:** *l*-, oxidation, tobacco leaf enzymes, *Tolbert, Clagett, and Burris*, 905  
*Phenyl-*. See Phenyllactic acid
- Lactobacillus bifidus:** Variants, nutrition, *Tomarelli, Norris, György, Hassinen, and Bernhart*, 879
- Lactobacillus leichmannii:** Vitamin B<sub>12</sub> determination, use, *Hoffmann, Stokstad, Hutchings, Dornbush, and Jukes*, 635
- Leuconostoc citrovorum:** Growth factor, *Sauberlich and Baumann*, 871  
 — —, urine excretion, folic acid effect, *Sauberlich*, 467
- Lipase:** Specificity, *Nachlas and Seligman*, 343
- Liver:** Acetoacetate formation from pyruvate, acetate, and fatty acids, *Crandall and Gurin*, 829  
 Enzymes, protein amino acids, relation, *Williams and Elvehjem*, 559  
 Fatty acids, oxidation, carbohydrates, effect, *Weinhouse, Millington, and Friedman*, 489  
 Glutamine deamidation, anterior pituitary growth hormone, effect, *Bartlett and Gaebler*, 529  
 Glycogen, formation, methionine, cystine, and cysteine effect, *Hess*, 23  
 —, octanoic acid conversion to, C<sup>14</sup>, C<sup>15</sup>-labeled octanoate use in study, *Lorber, Cook, and Meyer*, 475  
 3-Hydroxyanthranilic acid metabolism, *Schweigert and Marquette*, 199  
 Phosphorus compounds, acid-soluble, fractionation, *Sacks*, 655  
 Quinolinic acid formation from 3-hydroxyanthranilic acid, *Henderson and Ramasarma*, 687  
 Respiration, *Richert, Edwards, and Westerfeld*, 255  
 Xanthine oxidase, determination, *Richert, Edwards, and Westerfeld*, 255
- Lymph:** Nodes, cryoglobulin, lymphosarcoma, physical properties, *Abrams, Cohen, and Meyer*, 237

## M

- Melanin:** Chemistry, *Mason*, 803
- Methionine:** Liver glycogen formation, effect, *Hess*, 23  
*Mold:* See also *Neurospora*
- Monopalmitin:**  $\alpha$ -, pancreas, isolation, *Jones, Koch, Heath, and Munson*, 755
- Monothiophosphate:** Hydrolysis and oxidation, enzymatic, *Binkley*, 317
- Mucoid:** Ovo-, *Fredericq and Deutsch*, 499
- Muscle:** Adenylic acid, brain glycolysis, effect, *Greenberg*, 781  
 Protein, electrophoresis and ultracentrifugation, *Amberson, Erdős, Chinn, and Ludes*, 405  
 Vitamin E deficiency, effect, *Roderuck*, 11  
 — — —, transamination, *Barber, Basinski, and Mattill*, 17  
 See also *Diaphragm*
- Mutase:** Phosphoglyceric, reaction mechanism, *Sutherland, Posternak, and Cori*, 153

## N-

- Naphthoquinone(s):** Fungus germination, effect, *Foots, Little, and Sproston*, 481
- Neopyrithiamine:** Thiaminase, fish, effect, *Sealock and White*, 393
- Neurospora:** Nicotinic acid replacement, growth effect, *Henderson*, 677
- Niacin:** Requirement, fructose and carbohydrates, effect, *Hundley*, 1
- Nicotinic acid:** Kynurenine conversion to, *Kallio and Berg*, 333  
 Quinolinic acid replacement, growth, effect, *Henderson*, 677  
 Tryptophan conversion to, *Kallio and Berg*, 333
- Nitrogen:** Ammonium salts, utilization, amino acid synthesis *in vivo*, *Ross, Smith, Womack, and Shane*, 307  
 Isotopic, hydroxyproline metabolism, use in study, *Stetten*, 31  
 Storage, mechanism, *Bartlett and Gaebler*, 523, 529

**Nitrogen—continued:**

Urea, utilization, amino acid synthesis *in vivo*, *Rose, Smith, Womack, and Shane*, 307

**Nuclease:** Ribo-. See Ribonuclease

Spleen cathepsin, *Maver and Greco*, 861

Thymus cathepsin, *Maver and Greco*, 861

**Nucleic acid:** Ribo-. See Ribonucleic acid

**Nucleoprotein(s):** Thymus, hydrolysis, cathepsins, *Maver and Greco*, 853

**Nucleotide(s):** Dihydrodiphosphopyridine, phosphorylation, oxygen and, *Lehninger and Smith*, 415

Diphosphopyridine, extraction and purification, *Clark, Dounce, and Stoltz*, 459

Pentose, determination, spectrophotometric, *Dische*, 379

**O**

**Octanoate:** C<sup>14</sup>, C<sup>13</sup>-labeled, octanoic acid conversion to liver glycogen, use in study, *Lorber, Cook, and Meyer*, 475

γ-(C<sub>7</sub>)-labeled, conversion to acetate, *Crandall, Brady, and Gurin*, 845

**Octanoic acid:** Liver glycogen conversion to, C<sup>14</sup>, C<sup>13</sup>-labeled octanoate use in study, *Lorber, Cook, and Meyer*, 475

**Oligophrenia phenylpyruvica:** *Prescott, Borek, Brecher, and Waelsch*, 273

**Organic matter:** Non-volatile, determination, micro-, *Johnson*, 707

**Ovomucoid:** *Fredericq and Deutsch*, 499

**Oxazolidone:** l-5-Vinyl-2-thio-, turnip and *Brassica* seeds, thyroid, effect, *Astwood, Greer, and Ettlinger*, 121

**Oxidase:** Xanthine, liver, determination, *Richert, Edwards, and Westfeldt*, 255

**Oxygen:** Dihydrodiphosphopyridine nucleotide phosphorylation, electron transport and, *Lehninger and Smith*, 415

**P**

**Palmitin:** α-Mono-, pancreas, isolation, *Jones, Koch, Heath, and Munson*, 755

**Pancreas:** α-Monopalmitin isolation, *Jones, Koch, Heath, and Munson*, 755

Ribonucleic acid, *Kerr, Seraidarian, and Wargon*, 773

**Pentose:** Nucleotides, determination, spectrophotometric, *Dische*, 379

**Peptidase(s):** Carboxy-. See Carboxypeptidase

Dehydro-. See Dehydropeptidase

L- and D-, dehydropeptidase and, separation, *Price, Meister, Gilbert, and Greenstein*, 535

**Peptide:** Alga, marine, *Dekker, Stone, and Fruton*, 719

**Phenylalanine:** L- and D-, determination, microbiological, *Prescott, Borek, Brecher, and Waelsch*, 273

Metabolism, *Lerner*, 281

**Phenyllactic acid:** Determination, microbiological, *Prescott, Borek, Brecher, and Waelsch*, 273

**Phosphoglyceric mutase:** Reaction, mechanism, *Sutherland, Posternak, and Cori*, 153

**Phosphoric ester(s):** Biological, *Lampson and Lardy*, 693, 697

*Totton and Lardy*, 701

**Phosphorus:** Acid-soluble compounds, liver, fractionation, *Sacks*, 655

Mass 32, uptake, brain, Theiler's GD VII virus, effect, *Rafelson, Winzler, and Pearson*, 583

**Phosphorylation:** Anaerobic, α-ketoglutaric acid dismutation, *Hunter and Hixon*, 67

Dihydrodiphosphopyridine nucleotide and oxygen, electron transport, *Lehninger and Smith*, 415

α-Ketoglutaric acid oxidation and, *Hunter and Hixon*, 73

Oxidative, kidney, factors affecting, *Pardee and Potter*, 739

**Pituitary:** Anterior, growth hormone, kidney glutaminase, effect, *Bartlett and Gaebler*, 523

—, —, liver glutamine, deamidation, effect, *Bartlett and Gaebler*, 529

Diaphragm glucose uptake, *Park and Krahl*, 247

**Plant:** Hydantoinase, *Eadie, Bernheim, and Bernheim*, 449

**Polysaccharide(s):** Water-soluble, sweet corn, *Dvonch and Whistler*, 889

**Proline:** Hydroxy-. See Hydroxyproline

**Propanediol phosphate:** Synthesis, *Lampson and Lardy*, 697

**Protein(s):** Amino acids, liver enzymes, relation, *Williams and Elvehjem*, 559

Muscle, electrophoresis and ultracentrifugation, *Amberson, Erdős, Chinn, and Ludes*, 405

Nucleo-. See Nucleoproteins

**Proteolysis:** Enzyme, blood serum, purification and properties, *Remmert and Cohen*, 431

**Putrescine:** *Hemophilus parainfluenzae* growth, effect, *Herbst and Snell*, 47

**Pyruvate:** Acetoacetate formation, liver, *Crandall and Gurin*, 829

Carbon<sup>14</sup>-labeled, diaphragm, utilization, *Villee and Hastings*, 131

## Q

**Quinolinic acid:** Formation from 3-hydroxyanthranilic acid, liver, *Henderson and Ramasarma*, 687

Metabolism, *Henderson and Hirsch*, 667

*Henderson*, 677

*Henderson and Ramasarma*, 687

*Henderson, Ramasarma, and Johnson*, 731

Nicotinic acid as replacement, growth, effect, *Henderson*, 677

Urine, tryptophan and 3-hydroxyanthranilic acid effect, *Henderson and Hirsch*, 667

**Quinolinic acid—continued:**

Urine, tryptophan effect, *Henderson, Ramasarma, and Johnson*, 731

## R

**Ribonuclease:** *Kerr, Seraidarian, and Wargon*, 773

**Ribonucleic acid:** *Kerr, Seraidarian, and Wargon*, 761, 773

Determination, *Kerr, Seraidarian, and Wargon*, 761

Pancreas, *Kerr, Seraidarian, and Wargon*, 773

## S

**Serine:** Deaminase, *Escherichia coli*, *Wood and Gunsalus*, 171

**Sodium:** Alloxan diabetes, relation, *Grunert and Phillips*, 821

Biological fluids, determination, colorimetric, *Stone and Goldzieher*, 511

Blood serum, determination, colorimetric, *Stone and Goldzieher*, 511

Glutathione relation, *Grunert and Phillips*, 821

**Spleen:** Cathepsin, nuclease, *Maver and Greco*, 861

## T

**Tagatose-6-phosphate:** D-, synthesis and biological activity, *Totton and Lardy*, 701

**Tetanus:** Toxin, production, glutamine effect, *Mueller and Miller*, 39

**Theiler's GD VII virus:** Brain metabolism, relation, *Pearson and Winzler*, 577

—, phosphorus<sup>32</sup> uptake, effect, *Rafelson, Winzler, and Pearson*, 583

—, radioactive carbon from glucose, incorporation, effect, *Rafelson, Winzler, and Pearson*, 595

**Thiaminase:** Fish, neopyrithiamine effect, *Sealock and White*, 393

**Thiamine:** Neopyri-, thiaminase, fish, effect, *Sealock and White*, 393

**Thiol:** -Disulfide systems, oxidation-reduction potentials, *Freedman and Corwin*, 601

- Threonine:** Deaminase, *Escherichia coli*, <sup>21</sup> 171  
*Wood and Gunsalus*, 171
- Thymus:** Cathepsin, nuclease, *Maver and Greco*, 861  
 Nucleoproteins, hydrolysis, cathepsins, *Maver and Greco*, 853
- Thyroid:** 1-5-Vinyl-2-thiooxazolidone effect, *Astwood, Greer, and Ettlinger*, 121
- Tobacco:** Leaf, enzymes, glycolic acid and l-lactic acid oxidation products, *Tolbert, Claggett, and Burris*, 905
- Toxin:** Tetanus, production, glutamine effect, *Mueller and Miller*, 39
- Trypsin:**  $\alpha$ -Chymo-, inhibitors, *Kaufman and Neurath*, 623
- Tryptophan:** Metabolism, *Kallio and Berg*, 333  
 Nicotinic acid precursor, *Kallio and Berg*, 333  
 Quinolinic acid excretion, effect, *Henderson and Hirsch*, 667  
 Urine quinolinic acid, effect, *Henderson, Ramasarma, and Johnson*, 731
- Tumor:** -Bearing mice, uric acid and allantoin excretion, *Bass and Place*, 81
- Turnip:** 1-5-Vinyl-2-thiooxazolidone, thyroid, effect, *Astwood, Greer, and Ettlinger*, 121
- Tyrosinase:** Catechol oxidation, mechanism, *Mason*, 803
- Tyrosine:** Ketone body formation from, *Weinhouse and Millington*, 645  
 Metabolism, *Lerner*, 281
- U**
- Uracil:** Determination, colorimetric, *Soodak, Pircio, and Cerecedo*, 713
- Urea:** Nitrogen, amino acid synthesis *in vivo*, *Rose, Smith, Womack, and Shane*, 307
- Uric acid:** Excretion, tumor-bearing mice, *Bass and Place*, 81  
 Metabolism, gout, isotopic uric acid in study, *Benedict, Forsham, and Stetten*, 183
- Urine:** *Leuconostoc citrovorum* growth factor, folic acid effect, *Sauberlich*, 467
- Urine—continued:**  
 Quinolinic acid, tryptophan and 3-hydroxyanthranilic acid effect, *Henderson and Hirsch*, 667  
 —, —, effect, *Henderson, Ramasarma, and Johnson*, 731
- V**
- Veratramine:** Chemical constitution, *Jacobs and Sato*, 55
- Veratrine:** Alkaloids, *Jacobs and Sato*, 55
- Vinyl-2-thiooxazolidone:** 1-5-, turnip and *Brassica* seeds, thyroid, effect, *Astwood, Greer, and Ettlinger*, 121
- Virus:** Reproduction, biochemistry, *Kozloff and Putnam*, 207  
 Theiler's GD VII, brain metabolism, relation, *Pearson and Winzler*, 577  
 — — —, —, radioactive carbon from glucose, incorporation, effect, *Rafelson, Winzler, and Pearson*, 595  
 — — —, phosphorus<sup>32</sup> uptake, brain, effect, *Rafelson, Winzler, and Pearson*, 583
- Vitamin(s):** B<sub>12</sub> and related bacterial growth factors, chromatography, *Winsten and Eigen*, 109  
 —, determination, *Lactobacillus leichmannii* use, *Hoffmann, Stokstad, Hutchings, Dornbush, and Jukes*, 635  
 —, folic acid, and vitamin C, interrelation, chick, *Dietrich, Nichol, Monson, and Elvehjem*, 915  
 C, vitamin B<sub>12</sub>, and folic acid, interrelation, chick, *Dietrich, Nichol, Monson, and Elvehjem*, 915  
 E, muscle, deficiency effect, *Roderuck*, 11  
 —, —, —, transamination, *Barber, Bmsinski, and Mattill*, 17
- X**
- Xanthine oxidase:** Liver, determination, *Richert, Edwards, and Westerfeld*, 255
- Z**
- Zinc:** Blood, determination, trichloroacetic acid use, *Hoch and Vallee*, 205







